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Minimally processed baby leaf vegetables: Phytonutrient characterization and Nutritional Stability

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PhD Thesis

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Abstract

The consumption of fresh vegetables is globally recommended by worldwide health organizations as an effective mean to preserve and improve population's health status. The high concentration in certain bioactive phytochemicals normally present in vegetables is the reason for those recommendations, being the high intake of vegetables correlated to lower incidence of chronic diseases. However, in our modern society, the busy lifestyle of today's consumers does not allow to dispend a lot of time to prepare meals, being their decisions greatly influenced by the perceived convenience of a food product. Minimally processed vegetables become a convenient answer to those who want to include more fresh vegetables in the diet. The evolution in this market has been drove by the consumer demand for high quality fresh products that could deliver, in a convenient way, all the flavour and nutritional benefits associated to fresh vegetables. The minimally processed baby leaf products are one of the latest innovations in this sector, and permitted to add more variety, colours, formats and textures to the range of minimally processed vegetables previously available. These products are constituted by more immature leaves, being usually packaged whole, which grant to the product a different and appealing appearance. As leafy vegetables, they are seen as a healthy source of phytochemicals and showed great acceptance within consumers. However, it has been described that the nutritional quality of these products can be significantly influenced by different factors, like maturity stage and processing conditions. The main objective of this work was the evaluation of the nutritional quality of minimally processed baby leaf vegetables during shelf life, namely, vitamin, mineral, phenolic and carotenoid contents, that are the bioactive compounds normally associated to the health benefits provided by fresh vegetables. To achieve that, different extraction methods were used to extract the very different chemical structures from vegetal matrices. Several analytical methodologies were developed and/or applied, namely chromatographic methods coupled to diode array and mass detectors (vitamins, carotenoids and phenolics determination) and an atomic absorption spectrometry based method (mineral analysis), also coupled to a high resolution UV-Vis detector.

The results obtained showed that most of the main phytochemicals that contribute to the nutritional quality of baby leaf samples can suffer significant changes during shelf life. Among the micronutrients evaluated, the greatest changes were found among the vitamins, especially the water-soluble ones. However, the behaviour of each vitamin was strongly dependent on the sample species. Baby leaf vegetables were rich in vitamin C, provitamin A and vitamin E, being the new baby leaf, pea shoots, the richest sample on

vitamin C and provitamin A. Regarding the samples mineral content, the baby leaf studied revealed to be a good source of potassium and calcium, being their mineral content stable during storage. Organic wild rocket baby leaves were the sample with the highest mineral content. Among the different phytonutrients, the majority of the baby leaves studied showed a higher content of glycosylated flavonoid compounds. However, the phenolic profile of each sample was also greatly dependent on the species. Ruby red lettuce was the richest sample in phenolic compounds. Regarding carotenoid content, β-carotene and lutein, typical from green leafy vegetables, were also found in high amounts. Finally, from all the phytochemicals evaluated, it could be referred that the maturity stage of this young leaves did not represent a nutritional disadvantage, when comparing to the contents published for more mature leaves.

Keywords: baby leaf; micronutrients; phytonutrients; nutritional quality; HPLC-DAD-MS; HR-CS-AAS.

Resumo

O consumo de vegetais frescos é recomendado pelas organizações mundiais de saúde como um meio eficaz para preservar e melhorar a saúde pública. O elevado teor de fitoquímicos bioativos, normalmente presentes em vegetais, é a razão para essa recomendação, uma vez que um consumo elevado de vegetais tem sido associado a uma menor incidência de doenças crónicas. Contudo, o estilo de vida na sociedade atual não se coaduna com uma preparação demorada de refeições saudáveis e que incluam vegetais frescos em abundância. Atualmente, o consumidor baseia a sua decisão na conveniência que determinado produto alimentar pode oferecer. Os vegetais minimamente processados surgiram como uma opção conveniente para quem procura incluir mais vegetais frescos na dieta. A evolução neste mercado tem sido impulsionada pela maior procura de produtos frescos de alta qualidade e que, de uma forma conveniente, consigam preservar o sabor e os benefícios nutricionais, normalmente associados aos vegetais frescos. As ”baby leaf” são uma das mais recentes inovações do setor dos produtos minimamente processados. Estas surgiram como uma forma de acrescentar variedade, cor, formatos e texturas à gama de vegetais minimamente processados já disponíveis no mercado. Estes produtos são constituídos por folhas num estado de maturação precoce, sendo geralmente embaladas inteiras, conferindo aos produtos uma aparência diferente, o que tem atraído os consumidores. Como vegetais, estes são também reconhecidos como uma fonte saudável de fitoquímicos. No entanto, também é um facto que a qualidade nutricional dos vegetais pode ser significativamente influenciada por diferentes fatores, como o estado de maturação em que são colhidos e as condições de processamento a que são sujeitos. O principal objetivo deste trabalho foi avaliar a qualidade nutricional de vegetais “baby leaf” minimamente processados durante o seu prazo de validade. O estudo desenvolvido focou principalmente a presença de vitaminas, minerais, compostos fenólicos e carotenóides, que são os fitoquímicos bioativos normalmente associados aos benefícios do consumo de vegetais frescos. Durante a execução deste trabalho foram utilizados diferentes métodos de extração para obter, de matrizes vegetais, os diferentes compostos alvo. Foi necessário desenvolver e/ou aplicar várias metodologias analíticas para atingir o objetivo proposto, incluindo diferentes métodos cromatográficos acoplados a diferentes detetores (UV-Vis, MS) e um método de espectrometria de absorção atómica (análise mineral), também acoplado a um detector UV-Vis de alta resolução.

Os resultados obtidos mostraram que a concentração dos principais fitoquímicos que contribuem para a qualidade nutricional das “baby leaf” pode sofrer alterações

significativas durante a sua vida útil. Entre os micronutrientes avaliados, o teor de determinadas vitaminas foi o que registou as maiores variações, especialmente a concentração das vitaminas hidrossolúveis. No entanto, a evolução de cada vitamina mostrou-se muito dependente da espécie analisada. Os diferentes vegetais "baby leaf" analisados revelaram ser uma boa fonte de vitamina C, provitamina A e vitamina E. As folhas de ervilha, que foram recentemente introduzidas no mercado como um produto "baby leaf", foram a amostra com maior teor de vitamina C e pró-vitamina A. Quanto ao teor mineral das amostras, estas revelaram ser uma boa fonte de potássio e de cálcio. O teor dos diferentes minerais analisados mostrou-se estável durante o período de armazenamento estudado. As folhas de rúcula selvagem, de cultivo biológico, foram as que apresentaram o maior teor mineral. Relativamente aos diferentes fitonutrientes estudados, a maioria das amostras avaliadas revelou um elevado teor de flavonóides glicosilados. O perfil de compostos fenólicos de cada amostra foi também bastante influenciado pela espécie. As folhas de alface roxa foram, destacadamente, a amostra mais rica em compostos fenólicos. Quanto ao perfil de carotenóides, o β-caroteno e a luteína foram os principais compostos detetados nas "baby leaf", sendo a sua presença característica de vegetais de folhas verdes. Da análise de todos os fitoquímicos avaliados durante este trabalho foi possível verificar que o estado de maturação das "baby leaf" não representa uma desvantagem em relação à qualidade nutricional que é esperada neste género de vegetais, uma vez que os teores encontrados foram semelhantes aos reportados para folhas colhidas num estado mais avançado de maturação.

Palavras-chave: "baby leaf"; micronutrientes; fitonutrientes; qualidade nutricional; HPLC-DAD-MS; HR-CS-AAS.

Resumen

El consumo de vegetales frescos está recomendado por las organizaciones internacionales de la salud como un medio eficaz para preservar y mejorar la salud de los consumidores. Estas recomendaciones se basan en el elevado contenido en fitoquímicos bioactivos que normalmente se encuentran en alimentos vegetales. De hecho, un mayor consumo de verduras se ha correlacionado con una menor incidencia de enfermedades crónicas. Sin embargo, el estilo de vida en nuestra sociedad no es compatible con una larga y complicada preparación de comidas saludables y, actualmente, las decisiones del consumidor respecto a los productos alimentarios están muy influenciadas por la conveniencia que dichos productos puedan ofrecer. En este sentido, los vegetales mínimamente procesados se han convertido en una opción cómoda para aquellas personas que buscan para incluir más vegetales frescos en la dieta. La evolución en este mercado en los últimos años ha sido impulsada por la mayor demanda de productos frescos de alta calidad y que puedan ofrecer, de una manera conveniente, el sabor y beneficios nutricionales que normalmente se asocian con las verduras y los vegetales frescos. Las hojas “baby” son unas de las últimas innovaciones de productos mínimamente procesados que han ayudado a agregar más variedad, colores, formatos y texturas a la gama de vegetales mínimamente procesados. Estos productos se fabrican con hojas cosechadas en un estado más inmaduro, siendo generalmente envasadas enteras, lo que otorga al producto un aspecto diferente y que ha atraído los consumidores. Como otros vegetales, estos productos también son reconocidos como una fuente saludable de fitoquímicos. Sin embargo, también se ha descrito que la calidad nutricional de los vegetales puede estar influenciada de manera significativa por diferentes factores, como el estado de madurez y las condiciones de procesamiento. El objetivo principal de este trabajo fue la evaluación de la calidad nutricional de diferentes vegetales "baby" mínimamente procesados, durante su periodo normal de almacenamiento y distribución. Este estudio se centró principalmente en la presencia de vitaminas, minerales, carotenoides y compuestos fenólicos, que son los compuestos bioactivos que normalmente se asocian con beneficios para la salud que provienen del consumo de hortalizas frescas. Para lograr el objetivo propuesto, se utilizaron diferentes métodos de extracción, para obtener los diferentes compuestos de interés a partir de matrices vegetales. Varias metodologías analíticas fueron desarrolladas y/o aplicadas, incluyendo diferentes métodos cromatográficos acoplados a diferentes detectores (UV-Vis, MS) y un método de espectrometría de absorción atómica (análisis de minerales) también acoplado a un detector UV -Vis de alta resolución.

Los resultados obtenidos mostraron que la concentración de los principales fitoquímicos que contribuyen para la calidad nutricional de las hojas “baby” pueden sufrir cambios significativos durante su vida útil. Entre los micronutrientes evaluados, los mayores cambios se encontraron en las vitaminas, especialmente entre las vitaminas hidrosolubles. Sin embargo, la evolución particular de cada vitamina fue muy dependiente de la especie vegetal analizada. La mayoría de las muestras estudiadas presentaron altos contenidos en vitamina C, provitamina A y vitamina E. Las hojas de guisante, que han sido recientemente comercializadas como un nuevo producto "baby leaf", fueron los productos con el mayor contenido de vitamina C y provitamina A. El estudio de la fracción mineral de las hojas “baby” reveló que dichos productos son una buena fuente de potasio y de calcio, siendo su contenido mineral estable durante el almacenamiento. Las hojas de rúcola selvática de agricultura ecológica presentaron el contenido mineral más alto. Entre los diferentes fitonutrientes analizados, la mayoría de las hojas estudiadas mostró un mayor contenido de flavonoides glicosilados. Sin embargo, el perfil fenólico de cada muestra estaba también significativamente influenciado por la especie estudiada. El producto más rico en compuestos fenólicos fue el formado por hojas de lechuga rizada roja. Con respecto al contenido de carotenoides, β -caroteno y luteína fueron los principales en las "baby leaves", siendo su presencia característica en vegetales de hoja verde. Del análisis de todos los fitoquímicos evaluados durante el desarrollo de esta tesis fue posible verificar que el grado de madurez de las hojas "baby leaves" no se asocia a una desventaja en relación a la calidad nutricional, ya que sus niveles fueron similares a los que se apuntan en hojas más maduras.

Palabras clave: baby leaf; micronutrientes; fitonutrientes; calidad nutricional; HPLC-DAD- MS; HR- CS- AAS.

List of Publications

Publications in international peer-review Journals

1. Santos, J., Mendiola, J.A., Oliveira, M.B.P.P., Ibáñez, E., Herrero, M. 2012. Sequential determination of fat- and water-soluble vitamins in green leafy vegetables during storage. Journal of Chromatography A, Volume 1261, 179–188.

2. Santos J, Oliva-Teles MT, Delerue-Matos C, Oliveira M.B.P.P. 2014. Multi-elemental analysis of ready-to-eat “baby leaf” vegetables using microwave digestion and high-resolution continuum source atomic absorption spectrometry. Food Chemistry, Volume 151, 311-6.

3. Santos, J., Oliveira, M.B.P.P., Ibáñez, E., Herrero, M. Phenolic profile evolution of different ready-to-eat baby-leaf vegetables during storage. Journal of Chromatography A, *accepted*.

4. J. Santos, M. Herrero, J.A. Mendiola, M. T. Oliva-Teles, E. Ibáñez, C. Delerue-Matos, M.B.P.P. Oliveira. Assessment of nutritional and metabolic profiles of pea shoots: the new ready-to-eat baby-leaf vegetable. Food Research International. *Submitted*

5. J. Santos, M. Herrero, J.A. Mendiola, M. T. Oliva-Teles, E. Ibáñez, C. Delerue-Matos, M.B.P.P. Oliveira. Ready-to-eat salads: Adding fresh-cut aromatic herbs improves their nutritional value? LWT- Food Science and Technology. *Submitted*

Publications in Proceedings of Scientific Meetings

1. J. Santos, M. T. Oliva-Teles, C. Delerue-Matos, M.B.P.P. Oliveira. 2012. Avaliação da fração mineral de vegetais “baby leaf” prontos a consumir, ao longo do prazo de validade. 11º Encontro Química dos Alimentos, Actas, Bragança, Portugal.

Oral Communications in Scientific Meetings

1. Ready-to-eat green leafy vegetables: development of an extraction method to evaluate the antioxidant power.
   M. Almeida, J. Santos, M.F. Barroso, M. Correia, C. Delerue-Matos, M.B.P.P Oliveira.
   6th Meeting of Young Researchers of University of Porto (IJUP2013), 13-15 February 2013, Porto, Portugal.

Poster Communications in Scientific Meetings

1. Phenolic profile of pressurized liquid extracts from different ready-to-eat baby-leaf vegetables.
   Joana Santos, J.A. Mendiola, M.B.P.P. Oliveira, E. Ibáñez, M. Herrero
   SECyTA 2013 - XIII Scientific Meeting of the Spanish Society of Chromatography and Related Techniques, October 8-11, 2013. Tenerife, Canary Islands, Spain.

2. Could aromatic herbs add more nutrition and flavor to ready-to-eat salads?
   J. Santos, M. Herrero, J.A. Mendiola, M. T. Oliva-Teles, A. P. Vale, E. Ibáñez, C. Delerue-Matos, M.B.P.P. Oliveira.
   Workshop Advances in Molecular Structuring of Food Materials, 1-5 April, 2013. USP Pirassununga, São Paulo Brasil.

3. Determination of macro and micro mineral composition of ready-to-eat "baby leaf" vegetables by using microwave digestion and high-resolution continuum source atomic absorption spectrometry.
   J. Santos, M. T. Oliva-Teles, C. Delerue-Matos, M.B.P.P. Oliveira.
   Chemical Reactions in Foods VII, 14-16 November 2012. Prague, Czech Republic.

4. Nutritional value of pea leaves: the new baby leaf vegetable in ready-to-eat salads.
   J. Santos, M. Herrero, J.A. Mendiola, M. T. Oliva-Teles, A. P. Vale, E. Ibáñez, C. Delerue-Matos, M.B.P.P. Oliveira.
   Chemical Reactions in Foods VII, 14-16 November 2012. Prague, Czech Republic.

5. Avaliação da fração mineral de vegetais "baby leaf" prontos a consumir, ao longo do prazo de validade.
   J. Santos, M. T. Oliva-Teles, C. Delerue-Matos, M.B.P.P. Oliveira
   11º Encontro Química dos Alimentos, 16-19 September, 2012. Bragança, Portugal.

6. Development of HPLC-DAD-MS/MS methods for simultaneous determination of several fat- and water-soluble vitamins in green leafy vegetables.
   J. Santos, J.A. Mendiola, E. Ibáñez, M.B.P.P.B. Oliveira, M. Herrero (2011).
   13th instrumental analysis conference, 14-16 November, 2011. Barcelona. Spain.

7. Vitamin A content of Fresh-Cut Baby Leaf Vegetables used for salads during shelf life.
   J. Santos, M. Herrero, J.A. Mendiola, E. Ibáñez, M.B.P.P.B. Oliveira.
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8. Monitoring Quality of Fresh-Cut Baby Leaf Vegetables used for salads.
   J. Santos, M.B.P.P Oliveira
   Euro-Mediterranean Symposium for Fruit & Vegetable Processing, 18-21 April 2011 Avignon, France.

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List of Abbreviations

AAS	Atomic absorption spectrometry
ANOVA	Analysis of variance
APCI	Atmospheric Pressure Chemical Ionization
BHT	Butylated Hydroxytoluene
C*	Chroma
cfu	Colony forming unit
CV	Canonical Variate
d.w	dry weight
DAD	diode array detector
Depto	department
DPPH	1,1-diphenyl-2-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ESI	electrospray ionization
EtOH	Ethanol
EU	European Union
f.w.	fresh weight
FAO	Food and Agriculture Organization of the United Nations
FRAP	Ferric reducing/antioxidant power
FSV	fat-soluble vitamins
GC	gas chromatography
H	Hue
HACCP	Hazard analysis and critical control points
HPLC	High performance liquid chromatography
HR-CS-AAS	high -resolution continuum-source- atomic absorption spectrometry
i.d.	internal diameter
ICP	Inductively Coupled Plasma Spectrometry
IS	Internal standard
ISO	International Organization for Standardization
L* , a*, b*	Colour parameter

LC	Liquid chromatography
LC-MS	Liquid Chromatography Mass Spectrometry
LDA	Linear Discriminant Analysis
LOD	Limit of Detection
LOQ	Limit of Quantification
m/v	mass/volume
m/z	mass-to-charge ratio
MAP	Modified Atmosphere Package
MeOH	Methanol
Min	minutes
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTBE	Methyl tert-butyl ether
n.d.	not detected
N_2	Nitrogen
PAL	Phenylalanine ammonialyase
pH	Hydrogen ion potential
PLE	Pressurized Liquid Extraction
POD	Peroxidases
PPO	Polyphenol oxidases
RH	Relative humidty
ROS	Reactive oxygen species
RSD	Relative standard deviation
Rt, RT	retention time
SD	Standard deviation
sh	shoulder
TEA	Triethylamine
TPTZ	2,4,6-tris(1-pyridyl)-5-triazine
TSS	Total soluble solids
TTA	Total Titratable acidity
UV	Ultraviolet radiation
UV-Vis	Ultraviolet-visible
vs.	Versus
WHO	World Health Organization
WSV	Water-soluble vitamins
λ	Wavelength

CHAPTER 1.

Motivation, Aims and Thesis Outline

1.1 Thesis Motivation

Consumption of fruit and vegetables is increasing led by the known benefits of a balanced diet in the preservation of the human health (1, 2). However, the consumer choice among food products is greatly influenced by its sense of convenience. Minimally processed vegetables are presented as fresh, nutritious and convenient food products. They are ready to eat (i.e. do not need further preparation), are easily transported and have extended shelf life (2, 3).

In the fresh-cut market, consumers are always demanding for new products, with different colours, formats and textures. The fresh-cut baby leaf vegetables represent a successful reply to that demand, with great market results that are expected to be maintained or increased in the nearby future (4, 5). Baby leaf products are young leaves, harvested at early stage of maturation and in an active metabolic stage. Besides the novelty about their appearance (the baby leaves are packaged whole) and texture (normally more tender), as leafy vegetables, they are also presented to the consumer as a healthy source of phytochemicals that may improve or maintain health and wellbeing (5).

The study of the nutritional quality of baby leaf was the major motivation to the development of this thesis. Several known facts were taken into consideration when delineating this thesis project, from which the following were considered more critical:

1. Plants tissue remains alive throughout the shelf life;
2. Processing, though minimal, is known to cause the onset of many physiological changes, leading to a reduction in quality;
3. Maturity of the baby leaves is also a key feature that could influence the overall nutritional quality of these minimally processed products, as higher metabolic rates are associated to younger leaves.

The nutritional value of fresh-cut vegetables is something that the consumers cannot judge for themselves when buying the product, relying exclusively on the nutritional label provided by the producers. However, there is also a common knowledge that associates fresh vegetables with the presence of essential nutrients like dietary fibre, vitamins, minerals and also phytonutrients like carotenoids and phenolic compounds (2, 3). The vitamin content is normally one of the main dietary advantages of vegetable consumption. However it is also recognized that vitamins can be easily lost during processing and storage. Leafy vegetables are also a privileged source of minerals, but their content can be greatly affected by genetics, agronomic practices and maturity stage, which can be an

issue in the case of baby sized leaves. The thought associating fresh vegetables with antioxidant compounds is increasing among the consumers, and is usually related with their phytonutrient content. For this reason, the nutritional quality of fresh-cut vegetables is closely related to the presence of phenolic compounds and carotenoids, among other phytochemicals. The content and stability of these compounds is very dependent on different internal and external factors (6). In baby leaf vegetables, this subject has not been yet greatly discussed in the literature.

With the work developed during this thesis, we seek to obtain this information, about the nutritional quality of baby leaf products. Not only essential to ensure consumer trust in these products, but also to improve the producers knowledge about the behaviour of baby leaves metabolism during shelf life, which in turn is useful to finding ways to preserve their nutritional quality.

1.2 Research Aims

In this context, the main objective of this work was the evaluation of the nutritional quality of several minimally processed baby leaf vegetables during shelf life, especially the traits associated with the dietary advantages of vegetable intake, namely, vitamin, mineral, phenolic and carotenoid contents.

To accomplish the main objective, different specific objectives were considered throughout the study:

1. Development and validation of simple and/or environmentally friendly methods to extract vitamins, minerals, phenolic and carotenoid compounds from vegetable matrices;
2. Development and validation of HPLC-DAD-MS based methods for the identification and quantification of the different phytochemicals that contribute for the nutritional quality of baby leaf vegetables;
3. Development and validation of AAS based methods for the identification and quantification of the mineral content of baby leaf vegetables;
4. Study the evolution of each phytochemical content determined in several baby leaf samples along a typical storage period, simulating the market conditions;
5. Characterize and compare physicochemical characteristics as well as nutritional quality advantages of a new baby leaf product and new formulations of ready-to eat salads.

1.3 Outline of the thesis

According to the main objective of this PhD work, this thesis presentation was organised in eight different chapters. In this chapter the motivation, research aims and the thesis outline are described. **Chapter 2** presents an overview about minimally processed baby leaf vegetables, focusing on the main factors that affect their quality during shelf life. The main determinants of the quality of minimally processed vegetables were reviewed and discussed, with specially attention to the different components that contribute to the nutritional quality of these products. The Chapters 3 to 7 contain the main experimental results, distributed as follows:

In **Chapter 3** a new method to sequentially analyse fat- and water-soluble vitamins was establish, using an sequential ultrasound extraction and liquid chromatography methods coupled to diode array and a triple quadrupole mass detectors to identify and quantify different free forms of vitamins. The vitamin content was assessed in twelve fresh-cut green leafy samples before and after refrigerated storage to study the evolution of these compounds during product shelf life.

In **Chapter 4** a High Resolution - Continuum Source - Atomic Absorption Spectroscopy method for mineral analysis in vegetables was developed and validated to analyse the mineral content of eight baby leaf samples. The mineral profile of the samples was compare to the profile of more mature products. Also the mineral composition of a sample from organic production was compared to one cultivated in conventional agricultural system. The stability of the mineral content during storage was also studied. To compare the mineral composition of the different samples a multivariate statistical approach was followed.

In **Chapter 5** a pressurized liquid extraction method was optimized and applied to the extraction of phenolic compounds of eleven baby leaf samples. An HPLC-DAD-MS method was developed to identify and quantify different phenolic compounds. The levels of each identified compound were compared between the beginning and end of storage period to study the evolution of the different type of compounds in the different baby leaf samples.

In **Chapter 6** the nutritional value and the phytonutrient composition of pea shoots, a baby leaf recently introduced in the market, was thoroughly studied through different analysis methodologies. The nutritional quality of the pea shoots was evaluated by determining the macro (water, protein, fat, dietary fibre, ash and sugar content) and micronutrient composition (vitamins and minerals) and the phytonutrients content, namely

phenolics and carotenoids. The stability of the leaves quality was also assessed by comparing the nutritional components and several physicochemical at the beginning and end of storage.

In **Chapter 7** the nutritional quality of 4 fresh-cut aromatic herbs added to ready-to-eat salads mixtures, to improve flavour and quality, was monitored through shelf life. The nutritional advantages of this new formulation of leafy salads was assessed by studying the main components that contribute to the nutritional quality of the aromatic herbs (macro, micro and phytonutrient compositions), as well as, their antioxidant capacity. Different quality aspects related to colour and flavour were also addressed. The quality stability of the aromatic herbs during storage was also investigated.

Finally, **Chapter 8** presents the overall conclusions of the work developed, referring the main contributions achieved by this PhD thesis.

1.4 Work Plan of the Thesis

To accomplish the objective proposed in this thesis, the work developed was divided into four main phases, accordingly to the sequence presented in diagram displayed on Figure 1.1.

The work was mainly developed in three research laboratories:

- Laboratory of Bromatology and Hydrology, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto;
- FOODOMICS Laboratory at Institute of Food Science Research – CIAL (CSIC-UAM), Madrid;
- GRAQ-REQUIMTE, Department of Chemical Engineering of Instituto Superior de Engenharia do Porto, Instituto Politécnico do Porto.

The main experimental results obtained during the three initial phases are presented as scientific articles comprised within the chapters 3 to 7 of this thesis.

Phase 1: Sampling

Samples reception at the laboratory one day after minimally processed:

12 baby leaf fresh-cut samples

Green and red ruby lettuce | Swiss chard | Lamb's lettuce | Spinach | Pea shoots | Watercress | Garden cress | Mizuna | Red Mustard | Wild rocket *(current* and *organic)*

4 fresh-cut aromatic herbs

Chives | Coriander | Spearmint | Parsley

Samples divided in two groups:
1. **day 1** analyzed at the beginning of shelf life
2. **day 10** analyzed after 10 days of refrigerated storage

Phase 2: Fresh sample analyses

Sample preparation: fresh sample analysis and homogenization

Physicochemical analyses:
- -Colour
- -pH
- -Total soluble solids
- -Total Titratable Acidity

Macronutrient Evaluation:
- -Water
- -Protein
- -Fat
- -Dietary Fibre
- -Ash
- -Sugars (by difference)

Preparation of composite samples:
1. Freeze part of the samples at each sampling day
2. Proceed with the freeze dried process
3. Homogenization of the freeze dried samples in a knife mill
4. Stored protected from light, oxygen and heat until analysis

Phase 3: Freeze dried sample analyses

Bioactive Compounds Characterization

Extraction with ultrasounds

Vitamins (LC-DAD-MS/MS)

Carotenoids (LC-DAD-MS^n)

Antioxidant Capacity (DPPH and FRAP inibhition assays)

Microwave assisted digestion

Minerals (HR-CS-AAS)

Pressurized Liquid Extraction

Phenolic Compounds (LC- DAD-MS^n)

Phase 4: Results

Development of analytical methods and sample analyses

Paper I: Sequential determination of fat- and water-soluble vitamins in green leafy vegetables during storage

Paper II: Multi-elemental analysis of ready-to-eat "baby leaf" vegetables using microwave digestion and high-resolution continuum source atomic absorption spectrometry

Paper III: Phenolic profile evolution of different ready-to-eat baby-leaf vegetables during storage

Analyses of different components of selected fresh-cut leafy samples

Paper IV: Assessment of nutritional and metabolic profiles of pea shoots: the new ready-to-eat baby-leaf vegetable

Paper V: Ready-to-eat salads: Adding fresh-cut aromatic herbs improves their nutritional value?

Figure 1.1 Work plan executed during the development of this PhD thesis.

1.5 References

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CHAPTER 2.

Minimally processed vegetables:

the case of the fresh-cut baby leaf vegetables

This chapter provides an overview about minimally processed baby leaf vegetables, focusing on the factors that quality during shelf life, and also pointing out their main quality features.

2.1 Introduction

There is an increasing awareness regarding the role of a balanced diet in preserving and improving human well-being and health within our current society (1). Vegetables are a source of essential nutrients like dietary fibre, vitamins, minerals and also phytonutrients like carotenoids and phenolic compounds, crucial to human nutrition and health (2). Most of the health benefits attributed to the vegetable consumption are related with a high concentration in phytochemicals. Although the link between the role of vegetables in cancer protection has not been completely established, there is a consensual evidence of the benefits in the prevention and control of certain diseases or disorders (3, 4). For this, WHO and FAO recommend a daily consumption of vegetables should be increased to 400g, ideally to 800g (5). However the actual numbers are still below the recommendations in many countries (1). Within the several motives that have been pointed out as reasons for a low vegetable intake (eg. availability, lower income, less time for cooking, leisure instead of cooking and increasing out-of-home food consumption), the time required to prepare a meal with vegetables is one of the most referred by consumers with a busy lifestyle (1).

In today's society the choices related with food are significantly correlated with the need for convenience. As so, this is the main attribute that drives fresh-cut vegetable production. Convenience is related with reducing the input required from consumers when shopping for food, in preparing, cooking and cleaning after the meal, and can be defined in terms of the time, physical energy and mental effort savings offered to consumer in food-related activities (6). The minimally processed fresh-cut vegetables are the answer to consumers, as they combine more convenience and, at the same time, intake of healthy food in their daily lives (7).

2.2 Minimally processed vegetables

Nowadays, the worldwide fresh-cut industry is a multi-billion dollar sector, largely driven by increasing consumer demand for healthy, freshly prepared and convenient fruits and vegetables (8). The minimally processed vegetables maintain their fresh-like state and healthy characteristics, and require a minimal time of preparation before consumption, with the possibility of being consumed right out of the package (1, 9). According to the former International Fresh-Cut Produce Association the fresh-cut products are trimmed, peeled, washed, and cut into 100% usable product that is

subsequently packaged to offer consumers convenience, high nutrition, and fresh characteristics during shelf life (10). The extension of the fresh-cut product shelf life depends on a combination of a correct cooling treatment with optimal packaging conditions and good manufacturing and handling practices, throughout the entire product chain (11). The fresh-cut industry is continuously applying new technologies in order to extend the shelf life of these products, keeping the best sensory, microbial and nutritional quality and, above all, the safety of fresh processed vegetables. Besides convenience and nutritional value, the main advantages of these products to consumers and food services (restaurants and catering companies) are the reduced preparation time, the fresh–like characteristics, the uniformity and consistency of a high quality product, the easy supply of healthy products, the reasonable price and being easy to store, requiring little storage space and generating low quantities of waste (12).

The first appearance of these products in the market was in the 1940s and did not have the success that we see today. In those days, the vegetables used had poor quality for fresh-cut processing and the technology for optimal refrigeration throughout distribution and adequate packaging was not available (7). To become the successful growing sector that we know today several improvements had to be adopted, beginning with choosing high quality vegetables, improving the processing and packaging methods and also storage conditions. To avoid the appearance of visible defects (eg. discoloration, browning of the cut surfaces and flaccidity caused by loss of water), processors had to combine refrigeration with high quality raw products, gentle handling procedures and innovative packaging (8). With this measures the two main purposes of the minimal processing, namely the preservation of the fresh product, without losing its nutritional quality and achievement of a sufficient shelf life that could be feasible with distribution within the region of consumption, was attained (13). Today, the fresh-cut products available in the market are still evolving, with development being now focused on the importance of enhanced flavour, on providing a more diversity of products and making even better and appealing ready-to-eat vegetable products (7, 14). This way, the fresh-cut vegetables could be an option to consumers who want to add more vegetables in their meals, in a convenient way, adding also an excellent source of antioxidant compounds (eg. anthocyannins, phenolics, carotenoids, ascorbic acid, vitamin E) that could be beneficial to preserve health (2).

To maintain the fresh state of minimally processed vegetables for longer periods, moderate processing techniques and sealed pouches or trays packages are used to avoid contamination during storage and distribution (15). The raw plant tissue remains alive throughout the shelf life and the minimal processing causes the onset of many

physiological changes, leading to a reduction of quality and shelf life. To preserve quality and avoid the negative impact of those changes it is essential to understand the mechanisms that underlie changes of colour, texture and flavour (Figure 2.1). The enzymes released during processing, especially polyphenol oxidase (responsible for browning) and lipoxidase, (catalyses peroxidation reactions, originating aldehydes and ketones with unpleasant smell) are the responsible for biochemical degradation reactions of the minimally processed vegetables. The physiological responses of the vegetables to wounding and other minimal processing procedures include an increase in ethylene production, accelerating the ripening process, and increased respiration rate that causing a faster loss of quality (13).

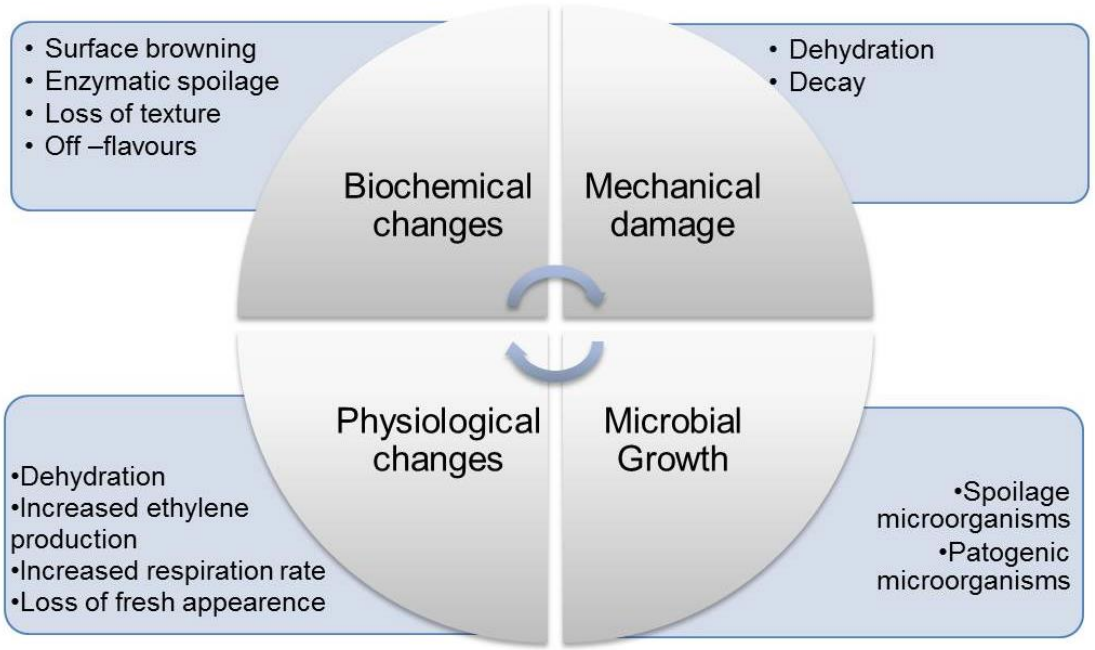

Figure 2.1 Factors that affect the deterioration and shelf life of minimally processed vegetables (adapted from Artés and Allende (12)).

Low storage temperature is the most important factor to control the decay of minimally processed fruit and vegetables. However, there are many other preservation techniques that are currently being used by fresh-cut industry, such as antioxidants, chlorines and modified atmosphere packaging (MAP). New techniques for preserving quality and inhibiting undesired microbial growth are being studied in all the steps of production and distribution chain. This evolution is of extreme importance as microorganisms are able to adapt to survive in the presence of previously effective control methods (16).

2.2.1 Microbiological Safety

The probability associated to pathogen survival and growth in minimally processed vegetables varies significantly with the type of product. Different factors like product's pH, presence of natural antimicrobials and/or competitive spoilage microflora, and respiration rate are known to affect pathogen survival and/or growth capacity. Most raw vegetables have high initial contamination levels which can easily exceed 10^6 cfu g^{-1} of viable microorganisms depending on a series of environmental circumstances (17). The contamination can be originated by a number of sources, during agricultural production (via animals or insects, soil, water), postharvest handling, processing (due to contaminated work surfaces/equipment, poor hygiene practices of workers), packaging (contaminated packaging materials/equipment) and distribution (8). Due to the nature of the minimal processing, a favourable environment and an extended time for spoilage microorganisms proliferation is created. When the colonizing spoilage species, such as *Pseudomonas* sp., lactic acid bacteria or yeasts reaches levels above 10^7 cfu g^{-1}, the contamination is easily detected by sensorial signs (accumulation of secondary metabolites like ethanol, lactic acid, ethyl acetate) (17). However, the microorganisms species can vary greatly according to the product or the type of packaging used (eg. the use of modified atmosphere may favour the development of different species) (18). Different psychotropic pathogenic species can develop at refrigeration temperatures or eventually in the case of breaking of the refrigeration chain In this case, In this cases, *Listeria monocytogenes, Aeromonas hydrophila* and *Clostridium botulinum* are the species that raise most awareness. Other pathogenic species, responsible for the emergence of new foodborne diseases are *Escherichia coli* O157: H7, *Salmonella* sp, *Yersinia enterolitica*, *Campylobacter jejuni* and some viruses and protozoa, whose presence in foods was associated to deficient agricultural and poor hygienic production practices (8). A common strategy to assure the safety of fresh-cut vegetables is the combined use of different barriers at lower individual intensities in order to delay the microbial growth. The different barriers can have additive or even synergistic antimicrobial effect, while their impact on sensory and nutritive properties on food is minimized. This combined used of several barriers is known as "hurdle technology" and is normally used in the production of minimally processed vegetables to obtain a gentle but effective preservation (19). Nonetheless, in all cases, prevention and sanitation are always the most important tools for keeping overall quality and safety of fresh-cut vegetables (20).

2.3 Baby Leaf Vegetables

In the actual fresh-cut market, the development of new varieties of raw materials has become necessary to fulfil consumer expectations in terms of convenience, freshness, flavour and quality (14). Consumers seem to appreciate softer textures, different tastes, shapes and colours. It was this demand for greater variety that promoted the introduction at a global level of “baby-sized leaves” (14, 21). Baby-sized leaves appear as one of the most promising fresh-cut development, being also presented as a natural source of health promoting bioactive phytochemicals (14). The market of pre-packed baby-leaf salad has showed great results for the last decade, and it seems likely that this trend will continue in the near future (22, 23).

The baby leaf salads are prepared with young leaves, harvested at early stage of maturation and in an active metabolic stage. The leaves are washed, mixed, and packaged as whole and have a shelf life of about 7 to 10 days post-processing, depending on the commodities (21, 24). The baby leaves have shown some advantages during processing over the traditional fresh-cut products (14). First, these leaves can be processed without any further cutting once the whole leaf is harvested and packaged. Higher process efficiency can be achieved in baby leaf salads due to a higher percentage of usable product, since the processing method is easier and faster. They have also a more attractive presentation because the leaves preserve their 3-D structure and have less signs of oxidation due to a smaller stem diameter. These traits suggest that baby leaves could have a relatively longer storage potential in terms of colour, nutritional and microbiological quality stability (14). On the other hand, respiration rate of more immature vegetables is usually higher than in more mature products, which may lead to a faster loss of quality (25). Higher respiration rate can result in a rapid loss of acids, sugars and other components that determine flavour quality and nutritional value (26). However, due to a lower degree of cutting combined with a low storage temperature, the respiration rate can be reduced and the adverse decay reactions delayed, making the baby leaves suitable for the fresh-cut industry (14).

The maturity stage is one of the key aspects of the baby leaf production and it is one of the main factors determining quality and their quality stability during postharvest handling and shelf life (2, 27). The maturity indicators in baby leaf production are mainly the leaf and petiole length, as well as, in some cases, the leaf width (27). Currently, available in the market there are several different baby leaves, from the most common varieties of baby leaf lettuces, to lamb’s lettuce (or corn salad), purslane, turnip tops or turnip greens, spinach, swiss chard and several leafy vegetables from the brassica family

as the case of watercress, mizuna, wild rocket, arugula (or rucola), garden cress, red mustard and tatsoi. The baby leaf can be sold in individual packages or in fresh-cut mixed salads. Each vegetable has a specific crop cycle that can vary between 20 and 75 days depending on the environmental conditions and desired stage of maturity (14, 24, 28-31). Some of the mentioned species have the capacity to regrow after the first harvest, and it is common to make more 2 or 3 harvests, within 10 to 30 days of interval, depending on the season (23, 32).

The quality and the behaviour of baby leaves during shelf life was already the subject of different studies aimed to establish the best agricultural and handling practices for the varieties already available and also for new species that may be introduced in the fresh-cut market with baby sized leaves (14, 22-24, 28, 29, 33-35).

2.3.1 Production of fresh-cut Baby leaf products

The selection of specific varieties for baby leaf production is on-going. Species are selected based on the taste, disease resistance and capacity to resist to physical stress caused by processing, maintaining an extended shelf life without developing water-logging, discolouration or decay due to injuries (36). The success of baby leaf salads is closely related to the optimization of farming methods, handling chain, washing equipment and packing. Normally, the farming practices are optimized according to the specie characteristics, trying to manage the harvesting, transportation and processing within the shortest period possible (see Figure 2.2) (36). Ideally, the minimally processed baby leaf salads will be ready for distribution within the next 24 hours that followed harvesting. During field operations there are several factors affecting the final quality and shelf life of the product. There is also an optimal period of the day for harvesting for each species, related to the leaf photosynthetic activity, with the normal diurnal changes of the biophysical properties of the cell walls (plasticity and elasticity) and with the carbohydrate accumulation (36).

Only high quality baby leaves have the required “processability” and are able to withstand the rigorous processing that includes harvest, transportation, washing, sanitisation, de-watering and packaging (see Figure 2.2) (22). Baby leaves must be handled carefully to avoid mechanical damage and water loss (37). In general, all operations have an impact on the final quality of the product, being the control of temperature during processing essential to preserve the product quality and safety (Figure 2.2) (12). The use of low temperatures slows down the metabolism by decreasing respiration, ethylene production, enzymatic processes and microbial activity (24).

However, it is also important to keep the temperature above the freezing point or it could damage the visual quality of the leaves.

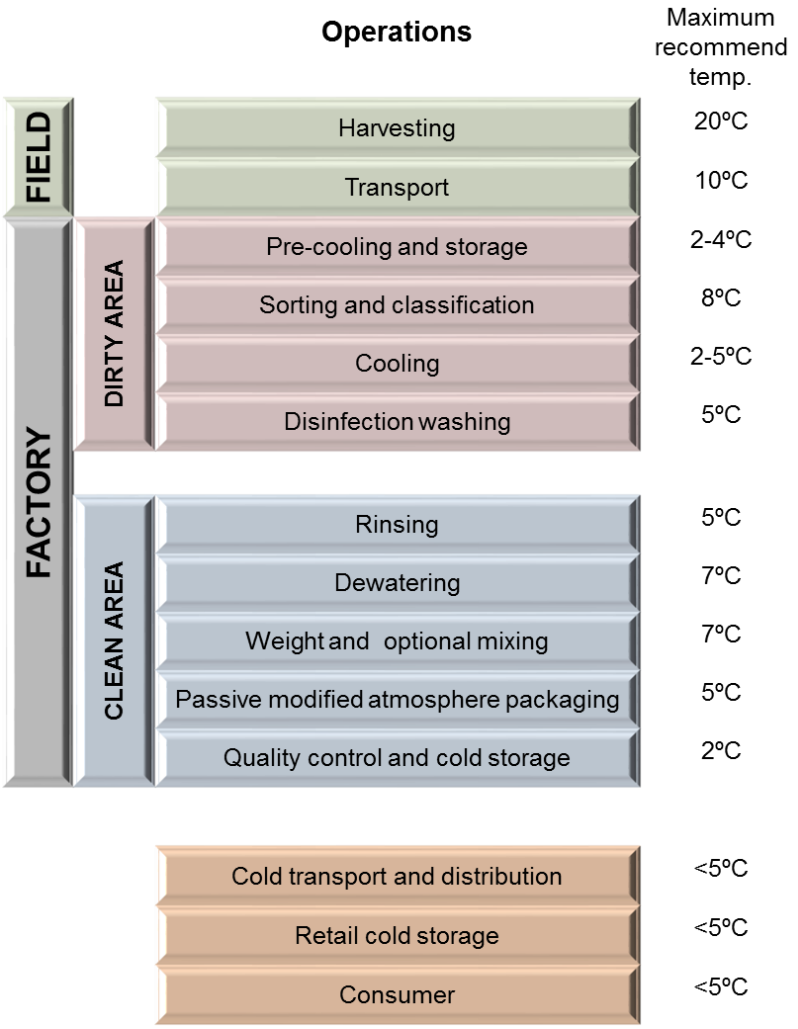

Figure 2.2 Diagram of the general unit operations in the minimally processing of baby leaves and maximum recommended temperatures to each processing step. (adapted from Artés and Allende (11)).

The relative humidity (RH) of storage is also a critical factor, as it can influence the microbiological population and the maintenance of acceptable visual quality (37). A RH of 95–98% is recommended for most of the leafy vegetables to avoid an excessive transpiration and water loss (38). The large surface area available for moisture loss of some baby leaves may favour an excessive transpiration when exposed to low RH conditions, that could lead to higher dehydration rates and consequently loss of visual quality, making leaves unfit to be processed. However, it was reported that baby leaf spinach exposed to lower RH (between 72 to 85%) in the pre-cooling and storage phase (Figure 2.2) had higher processability traits (37). The higher water loss makes the leaves stiffer, reducing the possibility of leaf damage during processing (22, 37). In the following

washing step, hydration of leaves was recovered, and the overall visual quality restored (21, 37). Light exposure during storage can also influence the leafy vegetables quality. Light may have a delaying effect on senescence by maintaining the photosynthetic activity of the tissue, and therefore its energy reserves and important antioxidant compounds (24, 39). However, there are contradictory results, once the effect of light seems to be dependent on several factors (type of vegetables, the duration of storage, the intensity and spectrum of the applied light and the type of packaging) (24, 39).

Most baby leaves available in the market are consumed raw in salads. Consequently, washing and disinfection are key steps to effectively reduce the initial microbial load of the leaves (16). The use of some chemical sanitizers during the washing step is a practical mean for achieving some decontamination. Damaged leaves are, normally, more susceptible to rapid growth of microorganism, including pathogens. Moreover, in immature leaves the microbial survival and proliferation could be enhanced by the high moisture content of uncut baby leaves (14). Because of this, this stage is essential to product's safety, being one of the primary elements of the postharvest sanitation program. However, as was stated earlier, it does not guarantee the total inactivation of human pathogens (31). Several works have been studying different methods to effectively reduce the contamination, avoiding the loss of quality and increasing safety of fresh-cut fruits and vegetables (17, 20, 40, 41), but until now, there is no single washing or disinfection solution to control all the parameters that maintain the safety, quality and shelf life for all minimally processed products (12, 16). Implementing a complete sanitation program that encompasses all the processing steps is essential to minimize the risk of contamination by pathogens and to assure consumers safety. This program implies that since the field until the product reaches the consumer house, all procedures follow the operational guidelines stated in Good Agricultural Practices (GAPs) (for suppliers of raw materials), Good Manufacturing Practices (GMPs), Standard Operations Procedures (SPOs) and an effective Hazard Analysis and Critical Control Points (HACCP) plan (13, 31).

Packaging is essential to preserve the quality of minimally processed products, avoiding further contaminations throughout the distribution chain. It also facilitates the produce handling during distribution, reduces loss of humidity, prevents spoilage and extends shelf life. The package enhances the convenience of the fresh-cut (it is easier to buy and transport) and attracts consumers as they evaluate packaging, labelling and product appearance in the purchase moment (9). Fresh-cut vegetables can either be packaged under atmospheric conditions (Passive Modified Atmosphere) or under a mixture of decreased oxygen and increased carbon dioxide (Active Modified atmosphere).

In both cases, leaves respiration decreases the oxygen concentration and rises the carbon dioxide levels until an equilibrium is reached, normally within a couple of days, depending on the storage temperature and the permeability characteristics of the package film. A low concentration of oxygen combined with low storage temperature is an effective method to delay the onset of senescence reactions. However, if extreme values of oxygen and carbon dioxide are produced inside the package, the beneficial effect of MAP can be easily lost (42). Generally, most of the baby leaf salads are packaged under atmospheric conditions (21).

According to the proposed operational diagram (Figure 2.2), the last operation before distribution is an effective quality control to guarantee the safety, suitability and compliance of the product specifications. Also the quality evolution of the fresh-cut baby leaf products during the next stage (distribution until consumption) is of utmost importance to success of the product. Consumers may try a new product if attracted by its appearance, but will not repeat the buy if the product fails to deliver an acceptable flavour and/or an appropriate texture (8, 9). A positive nutritional image is becoming increasingly valued by consumers, and may contribute to continued purchases. As minimally processed fresh-cut products, is expected that the visual and nutritional quality of the baby sized leaves changes during shelf life. However, the effect of the maturity stage in the overall quality of the leaves and especially in their phytochemical contents is not yet clear for most species.

2.4 Quality Assessment of Minimally Processed Baby Leaf Vegetables

During the postharvest period (from harvest until consumption) the vegetables suffer several biological changes. However, the technology applied in minimal processing has the purpose to enhance positive changes and delay the onset of negative senescence signs, extending the high quality period of the product. Different factors influence the composition and quality of vegetables (Figure 2.3). These include genetic factors (mainly represented by species and cultivar varieties), pre-harvest environmental factors (climatic conditions and agricultural practices), maturity at harvest, harvesting method (manual or mechanic) and post-harvest handling procedures (2). The genetic information (also called biotic or internal factors) of the plant has a great impact on the initial quality at harvest and how it responds to processing and storage environment. The selection of the more appropriate genotypes is crucial to guarantee a high quality product and also a good production yields. On the other hand, the environmental factors (also called abiotic or external factors) can inhibit or exacerbate the manifestation of the plant genetic

information. The effect of external factors influences the plants morphology, the physiological and biochemical defence mechanisms and the stress-induced senescence reactions (43). The impact of environmental factors on the products' quality are managed by the implementation of the best agricultural and handling procedures and also by using suitable technologies (e.g. packaging and refrigeration), thus avoid triggering the physiological senescence mechanisms (2, 43).

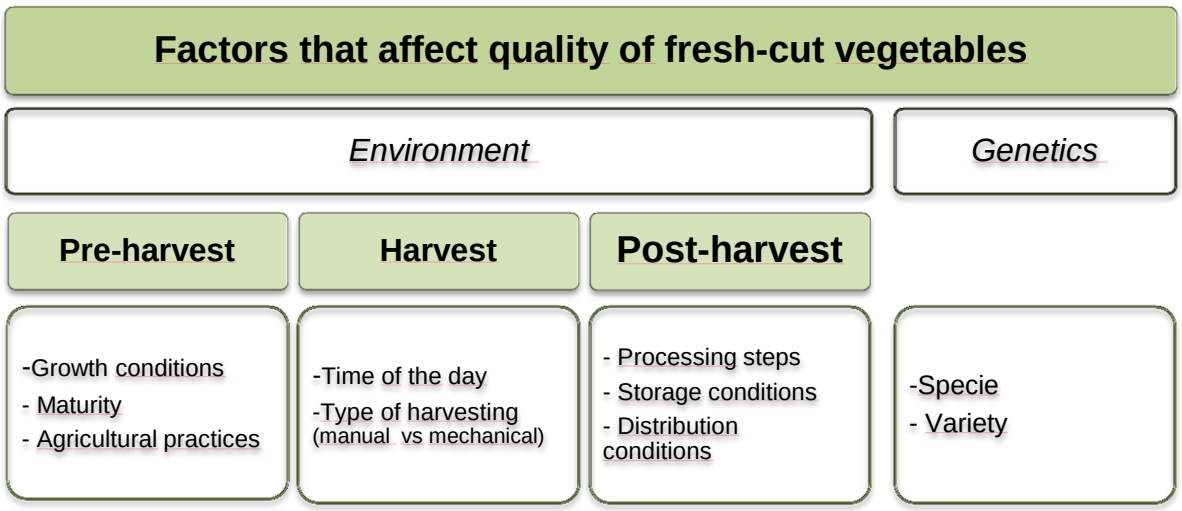

Figure 2.3 Factors that can affect the final quality of fresh-cut vegetables (adapted from Vicente et al. (44)).

Quality is a requirement in the actual society, but it is also a complex concept that involves many different aspects. Several definitions have been proposed and one of the most known is stated in the quality management standards from the ISO 9000 series, where is defined as "all the characteristics of the product (or service) that are required by the consumer" (45). "Quality" is also mentioned as a degree of excellence, a high standard, or value (9). The various attributes that define the quality depend directly on the type of product, the intended use and the preferences of the consumer. In this sense, the quality is determined by different characteristics and from combinations of those attributes (46). In minimally processed vegetables the most important attributes in the moment of purchase are "freshness" (consumers frequently associate the shelf life date as an indicative of healthiness and nutritional value), shape and colour of the product (1). At a consumption moment, consumers also state "freshness" as the main quality attribute. However, in this moment it is also associated to other sensory attributes, like texture and flavour (1). Therefore, freshness is the most important quality attribute of high quality vegetables, but it is also a concept difficult to define as it is not related to any single sensory attribute (47). Normally, consumers identify a product as "fresh" when it was

recently made or, in the case of vegetables, recently harvested. The appearance, texture, flavour, and nutritional composition of the vegetables are the attributes that contribute to the “freshness” concept preferred by consumers (1, 8).

The perception of the quality of a food product can only, in its nature, be performed by humans, since it is the way to apprehend which quality attributes are relevant to consumer acceptability (9). However, the operational costs of sensorial analysis are high and require a complex logistics to train sensory panels. These panels need extensive training to produce highly valuable results. Therefore, this method is more used in the development of new products and to establish the quality standards of a new product. In a routine basis, or even for research and commercial applications, analytical techniques are preferred to objectively monitor and quantify the quality changes occurring in the post-harvest phase (9, 48). Different instrumental measurements and techniques can be used to determine colour, appearance, flavour, texture, and nutritional quality with accurate and precise results (8). The results of instrumental tests can generally be related to chemical and physical properties, which allow inferring about the nature of the observed differences. Instruments tend to be more sensitive to small differences between samples and may be able to detect trends in quality loss before they can be detected by humans. Instrumental measurements can also provide a common language among researchers, industry and consumers (48). Rapid analytical techniques are favoured due to the fact that numerous samples are needed to cover the natural variation of biologic systems. This will lead to a better understanding of behaviour of vegetables during storage and how it can be affected by the storage conditions. Additionally, rapid analytical methods are interesting for the post-harvest industry for quality control operations (9). However, the challenge when measuring quality trough analytical methods is the need to relate the obtained results with the characteristics that really determine the consumer acceptability (9).

2.4.1 Colour

Appearance is by far the main factor affecting consumer choice and preferences (8, 9, 49, 50). Fresh-cut vegetables attract consumers if look as if is freshly cut, with a bright colour surface, and free of defects and decay (8). Colour is usually the first characteristic consumers rely to evaluate the vegetable quality, creating expectations about the flavour and texture, based on the identification of an ideal colour for that vegetable (51). The vegetable colour results from chemical, biochemical, microbial and physical changes occurring during growth, maturation, post-harvest handling and processing (49). Colour

measurement of vegetables has been used as an indirect measure of other quality attributes such as flavour and pigment contents, because it is simpler, faster and correlates well with other physicochemical properties (49). Plant pigments (chlorophylls, carotenoids, flavonoids and betalains) provide the chemical basis for vegetable colour (see Table 2.1). The chemical form of each pigment and the presence and state of non-pigmented substances can be affected by different factors influencing the human perception of colour as well as its intensity and stability (52).

Table 2.1 Characteristics of natural pigments in vegetables (adapted from Kidmose and Edelenbos (52)).

Pigment Group	*Colour*	*Approximate nº of compounds*	*Affected by*	*Molecular Structural type*
Fat-soluble pigments:				
Chorophylls	Green, Olive Brown	<50	Heat, pH, metals	Porphyrins
Carotenoids	Yellow, Orange, Red	>600	Light, oxygen, pH, heat	Carotenes and Xanthophylls
Water soluble pigments:				
Flavonoids	Yellow	>600	Oxygen, low pH, heat	Flavones and Flavonols
Anthocyanins	Red, Blue	<150	pH, heat, light, metals	Anthocyanidins
Betalains	Red, Yellow	<10	Heat, high pH, metals	Betacyanins and Betaxanthins

There are metabolic pathways responsible for colour development related with ripening or senescence still active after harvest. In addition, responses to mechanical, physical, chemical, or pathological stresses can also lead to colour changes during post- harvest phase. The development of a yellowish tonality in green vegetables is the result of normal senescence reactions, while browning, normally results from melanin formation by enzymes release in wounded vegetal tissues (51). Both phenomena are recognized by the consumer as loss of quality (49). In baby leaf vegetables, the colour is mainly determined by the presence of chlorophylls a and b. Chlorophylls are Mg^{2+} porphyrins molecules, and are the most abundant tetrapyrrole molecules in plants, involved in photosynthetic light-harvesting and energy production. However, these molecules are relatively unstable in processed green vegetables. The degradation of the chorophylls leads to colour changes, characterized by loss of green colour that consumers associated to “freshness”. Chlorophyll degradation is the major symptom of senescence, and is considered as decrease of quality in leafy vegetables (53). Chlorophylls can degrade into a series of derivatives with olive-brown (like pheophytins, pheophorbides,

pyropheophytins and pyropheophorbides) and green colours (like chlorophyllides, pyrochlorophylls hydroxychlorophylls), or may simply be degraded to colourless substances. A proposed mechanism for the degradation of chlorophylls in green vegetables is given in Figure 2.4 (52). The breakdown of chlorophyll was linked to the reuse of proteins in the plant and to protein degradation during senescence of living plants (54).

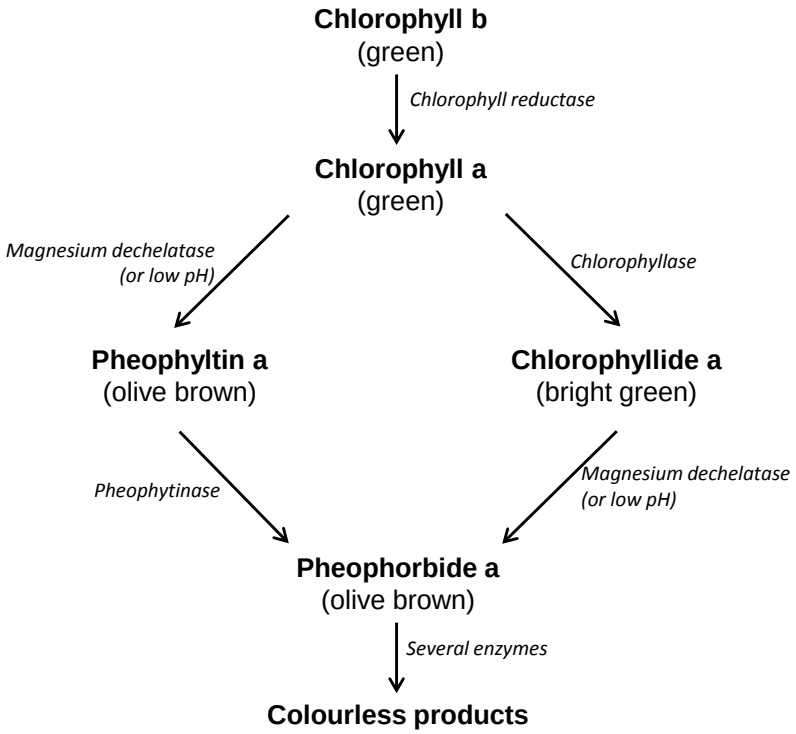

Figure 2.4 Schematic representation of initial stages of chlorophyll breakdown pathways in intact green plant tissue. The reactions occur in the chloroplasts (adapted from Kidmose and Edelenbos (52), Toivonen and Brummell (50)).

The vegetable colour can be subjectively or objectively measured, using the human eye or specific instruments, respectively. Colour charts or guides can be used as references for visual evaluation; however, results may be affected by human error and evaluation conditions (8). On the contrary, instrumental techniques attempt to describe colour mathematically in terms of human perception. One of the most common methods to evaluate colour is based on light reflected off or transmitted through the commodity (8, 51). Each vegetable pigment (Table 1) corresponds to a primary colour, red, blue and green, and colour may be further characterized by determining lightness (L*) or degree to which an object reflects light, and chroma (C*) or saturation, which is the intensity of colour or difference from grey at the same lightness (9). Some of the most popular systems are RGB (red, green and blue), mostly used in colour video monitors; Hunter L a

b, Commission Internationale de l'Eclairage's (CIE) L*a*b*, CIE XYZ, CIEL*u*v*, CIE Yxy, and CIE LCH. These differ in the symmetry of the colour space and in the coordinate system used (49). Of the many colour scales developed, the opponent colour system CIE L*a*b* is the most frequently used, based on L*, a*, and b* parameters and their derivative measurements (hue and chroma) (Figure 2.5) (51).

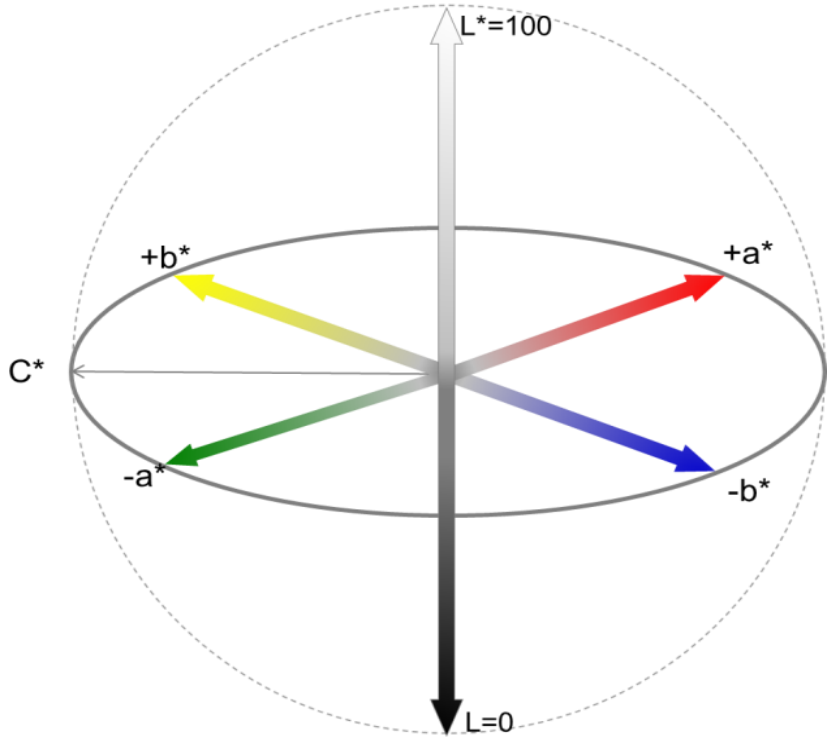

Figure 2.5 The CIE L*a*b* colour space. (0 (black) <L* (lightness) <100 (white); green <a*< red; blue <b*<yellow; C* (saturation); Hue is the location around the circumference) (Adapted from Pathare et al.(49)).

These numbers are plotted in a three-dimensional space (Figure 2.5), such that each colour has a distinct point in the CIE colour solid scale. Accordingly, ''a*'' represents red to green tonalities and ''b*'' represents yellow to blue. To approximate those coordinates to the colour perceived by humans, the a* and b* data should be transformed to the colour functions of hue (H) and chroma (C*). Hue, the colour name (red, orange, yellow, green, blue, purple etc.), is expressed as the angular distance from the a* axis, while chroma (brightness) is expressed as the distance from the origin (0,0) (51).

2.4.2 Flavour and Texture

After evaluating the appearance, the consumer evaluates flavour and texture of fresh-cut vegetables. This phase has a great impact in the success of the product, where the presence of unpleasant sensations or tastes can lead to the product rejection.

The flavour of vegetables is the result of complex interactions between perceptions of non-volatile and volatile compounds and mouth sensation (8, 55). The perception of basic tastes (sweet, sour, salty, and bitter) is very important for vegetables quality evaluation. Sweetness varies considerably in vegetables and is typically due to glucose, fructose, sucrose, and other sugars. However, its perception is influenced by sourness and aroma compounds. The sourness is also important to flavour quality of many vegetables and is due to citric, malic, acetic, and other organic acids. Loss of acids due to respiration changes the sugar/acid ratio increasing the sweetness sensation in these vegetables (8, 55). Saltiness is due primarily to sodium and potassium content of the vegetables. Bitterness can be determinant in the acceptance of certain vegetables and is due to many different compounds, including phenolic compounds, alkaloids or glucosides (9). Besides these basic tastes there are other sensory properties often mentioned as vegetable tastes: astringency (due to the presence of tannins) and pungency (55). Astringency can be defined as a drying or puckering mouth feel, generated by a complex reaction between certain polyphenols and proteins of the mouth and saliva (56). The aroma compounds contribute to flavour directly or by retronasal stimulation during chewing. In vegetables, these correspond mainly to nitrogen and sulphur compounds (8). Besides the volatile compounds formed in intact vegetables, the physical disruption of tissues (i.e., mastication) results in the production of others volatile compounds that also contribute to the vegetable flavour. The action of certain enzymes will also be determinant to the liberation of aroma compounds during mastication in several vegetables.

Maturity at harvest is a decisive factor in the flavour of all vegetables, as ripening process is characterized for a higher accumulation of sugars and a decrease in the acid concentration. Also the potassium and sodium accumulation in leaves and the content of several phenolic compounds can be greatly affected by the stage of maturity and by several environmental factors (34, 57). The concentrations of aroma compounds formed during maturation and ripening changes substantially (55). During the post-harvest phase, the vegetables flavour continue to evolve due to normal metabolism of the plant. In this stage, temperature and storage period have a major impact in the final flavour of the product. The development of desirable ‘‘typical’’ flavours can occur due to maturation process, but the development of “off’’ flavours during postharvest storage is one of the main concerns of fresh-cut producers. For example, the production of unwanted volatile compounds as ethanol and acetaldehyde can occur if the package used induce anaerobic metabolism. Some volatiles can be produced as a consequence of tissue starvation, as carbohydrate reserves are consumed in postharvest storage. Or, in the particular case of spinach baby leaves, the protein catabolism produces ammonia compounds (58).

The complexity of human perception of flavour and the interactions between many compounds difficult the characterization and quantification of flavour using instrumental techniques. It is also difficult to find the correct relationship between measurement of individual components and consumer acceptability. The use of sensorial methods is critical to this particular quality attribute (8, 9). However, there are some characteristics of flavour that may be determined instrumentally. Sweetness can be approximated by HPLC determination of individual sugars, or more rapidly but less accurately by the determination of total soluble solids with refractometer or hydrometer. Sourness may be evaluated by the pH or more accurately by measurement of total titratable acidity. The determination of sodium and potassium content can give an approximation of saltiness, while bitterness can be indicated by the content of phenolic compounds, especially tannins that are responsible for astringency sensation. The aroma volatiles may be very accurately measured using gas chromatography (9).

Consumers expect fresh-cut vegetables to feel firm and crunchy. However, in vegetable tissues the natural senescence reactions, water loss and wounding effects cause the softening and flaccidity, limiting the product shelf life (8). The texture of vegetative tissue is dependent on the composition of the cell wall, their turgor pressure and the middle lamella that connect individual cells (9, 59). The vegetable tissues contain a significant amount of water and other liquid-soluble materials surrounded by a semi-permeable membrane and cell wall. Approximately 90% of the dry matter of cell walls in edible vegetable tissues corresponds to different polysaccharides (mostly cellulose, hemicellulose, pectic polysaccharides), and the remaining 10% consists of simple phenolic compounds (lignin and small quantities of simple phenolic acids esterified to cell-wall sugars, particularly arabinose and galactose) and proteins/glycoproteins (most glycosylated, and many contain hydroxyproline) (59). Firmness depends on the physical anatomy of the tissue (like cell size, shape, cell wall thickness and strength), and on the extent of cell-to-cell adhesion, together with turgor status (25). In leafy vegetables such as lettuce, spinach, and brassica leaves, most of the edible tissues consist of epidermal tissues and a palisade of mesophyll cells, all of which are non-lignified. This structure in conjunction with the cellular influx of water due to osmosis produces the turgor pressure that responsible for maintaining the tissues rigid (turgid) 59. In small cells there is a tendency to have thicker cell walls, a lower relative amount of cytoplasm and vacuole, a greater area of cell-to-cell contact, and low amounts of intercellular air spaces, making the tissue firmer. Cell wall thickness and strength are major contributors to firmness, being normally determined by genetic factors (25). A baby sized leaf has mainly primary cell walls in the expansion phase. At this stage, the cell walls are not fully developed, and the

tender texture of baby leaves can also suffer a faster loss of texture properties after harvest (14).

The textural properties of the fresh-cut leaves can be affected by pre- and post-harvest handling conditions. Changes are, almost undetectable in the overall composition of the leaves, but they certainly have a dramatic effect on quality. Changes in the structural carbohydrates may be caused by both biosynthetic and senescence reactions, being necessary to slow the respiration rate and metabolism of the fresh-cut products to preserve the desired texture properties. Water loss due to transpiration process is also crucial for the texture of the leafy vegetables. A water loss of 2 to 3% during storage causes severe damage in the leaves, like signs of wilting, and loss of firmness and crispness. Furthermore, water loss induces physiological stress, which accelerates senescence, and consequently a higher membrane disintegration and leakage of cellular contents (60). However, even when dehydration is prevented, the natural maturation process still causes modifications at cell wall level and cause a natural evolution of the texture properties (50). During senescence phase, the compartmentalization of the cell begins to fail, due to changes in membranes, cell walls, subcellular organelles, proteins, and eventually ends in cell death. To postpone these events, a strict control of the storage temperature is necessary to diminish the respiration rate, the metabolism and also the transpiration process (50, 59).

In the case of fresh-cut vegetables, the texture evaluation is not easily assessed due to the great heterogeneity of the leaves, conferred by the presence of photosynthetic and vascular tissues with different textural properties (25). However, there are some aspects of texture that can be quantified objectively by sensorial and instrumental methods, particularly related to mechanical properties, like firmness. The instrumental methods applied to texture assessment are normally divided in three classes: fundamental, empirical and imitative tests (9). The more rapid and simple tests are normally the empirical, like the puncture, compression, extrusion, shear tests, which measure one or more textural properties. These are the most commonly used in quality control applications. Although these are destructive tests they have shown a good correlation with sensorial analysis of firmness properties (9). On the other hand, the measure of “crispness” by instrumental methods is more difficult (25).

2.4.3 Nutritional and Bioactive Compounds

The recognized nutritional value of fresh-cut vegetables is a hidden quality characteristic that the consumers can not perceive visually, but are expecting to get when

buying fresh-cut vegetables (1, 9). The appearance and nutritional value are the main quality factors influencing consumers at the purchasing moment (1). To increase the importance of the nutritional value to consumer's choice also contributes the recognition that different nutrients are not normally consumed individually, but as combined constituents of an adequate diet. Epidemiological surveys suggest that the total diet, thus the combination of multiple nutrients, has a greater influence on health than single specific compounds (2, 44). Vegetables are known to be good sources of dietary fibre, many vitamins and minerals, and some beneficial phytonutrients such as phenolics and carotenoids, all recognized as having a positive effect on human health. This knowledge together with the fresh-cut industry claims of convenience and healthiness, make the fresh-cut products a easy way to fulfill the dietary needs for fresh food (25). Nutritional labels on the product package are the only way to consumers distinguishes between a high or low nutrient content of different fresh-cut vegetables. However, the several changes that occur in the vegetable metabolism during harvesting, handling and processing can affect their chemical composition (25). Genetics, growing conditions (light, temperature and season), production practices (fertilization, irrigation) and maturity stage will, also, affect the nutritional value (9).

2.4.3.1 Nutritional compounds

Nutritional compounds are molecules that the human organism needs for the normal physiological and metabolic processes. They are divided in macronutrients (carbohydrates, fats, dietary fibre, proteins and water) and micronutrients (vitamins and minerals) (44).

2.4.3.1.1 Macronutrient Composition

The main macronutrient composition of leafy vegetables is similar for a great number of species. About 90% of the total mass of the leaves correspond to water. Small variations (5-8%) between different vegetables can occur due to structural differences. The other 10% of the composition correspond to the compounds that represent the nutritional advantages of consuming fresh leafy vegetables. These vegetables are normally recognized as good sources of dietary fibre, showing, generally, a mean content in the range of 1% to 3% of the total fresh weigh (f.w.) (44). The dietary fibre is mainly derived from cellular walls of plants, being composed by macromolecules such as cellulose, hemicellulose, pectins, lignin, resistant starch and others non–digestible polysaccharides. The principal health benefit of a dietary fibre intake is related to the modulation of bowel function, the ability to reduce cholesterol and the risk of coronary heart diseases, reduced type II diabetes and improvement of weight maintenance (61).

Normally, meals that have a high content of dietary fibre promote the sensation of satiety earlier, and are usually relatively low in calories. In most of the leafy vegetables the dietary fibre content is normally enough to legally print on the label the claim 'source of fibre', which represents a content of 3 g of fibre/100 g or 1.5 g of fibre/100 KCal (62). Regarding the other macronutrients, the leafy vegetables can have a protein content in the range of 1-4%, showing also low amounts of available carbohydrates (<3%) and fat (<0.5%) (44).

During storage under proper refrigerated conditions, the macronutrient composition of the vegetables is usually stable (9). However, some slight changes can occur in dietary fibre fraction. The action of several enzymes can alter the solubility and molecular size of the cell wall constituents. These changes can reduce the quality of the product once they have an impact the structural composition of the cell wall, and lastly the textural properties of the leaf (see section 2.4.2) (44).

In a routine basis, the determination of the macronutrient composition of leafy vegetables is performed using official chemical methods published by regulatory entities or by the AOAC INTERNATIONAL organization. For example, the protein content of vegetables is calculated by multiplying the total nitrogen content by a factor of 6.25 (AOAC method 920.54), while soluble dietary fibre in plant tissues may be quantified in a phosphate buffer extract using an enzymatic-gravimetric method (AOAC method 985.29) (63).

2.4.3.1.2 Vitamins

Vitamins are classically defined as a group of organic compounds required in very small amounts for the normal development and functioning of the body. They have several biochemical functions and an important role in the protein metabolism, maintenance of blood glucose levels, and regulation of cell growth and differentiation. They must be obtained through dietary sources as they are not synthesised by the body, or only produce in insufficient amounts. The 14 vitamins known today are divided into two categories: the water-soluble vitamins (WSVs) including the B group vitamins (B_1, thiamine; B_2, riboflavin; B_3, niacin; B_5, pantothenic acid; B_6, pyridoxine; B_9, folate/folic acid; B_{12}, cyanocobalamine; biotin; and choline) and vitamin C; and the fat-soluble vitamins (FSVs), including vitamins A, E, D, and K (44, 64). The principal vitamins of leafy vegetables and their functions in the body are presented in Table 2.2. All vitamins have different functions and their content may vary greatly within different species of vegetables. Different contents can also occur within cultivars, as well as between different lots of the same cultivar grown under different environmental conditions (2).

Table 2.2 Major functions of the principal vitamins found in leafy vegetables (adapted from Kader (2), Northrop-Clewes and Thurnham (66)).

Vitamin	Major function in the body
Vitamin A	Essential for normal vision; promotes bones and tooth development; important for gene expression, reproduction, embryonic development, growth and immune function.
Vitamin E	Antioxidant action; protects vitamins A and C and fatty acids from oxidation; prevents damage of cell membranes.
Vitamin K	Helps blood to clot; may play a role in bone health.
Vitamin C	Formation of collagen, wound healing; maintaining blood vessels, bones, teeth; absorption of iron, calcium, folate; production of brain hormones, immune factors; antioxidant action.
Thiamin (B_1)	Helps release energy from foods; promotes normal appetite; important in nervous system functioning.
Riboflavin (B_2)	Helps release energy from foods; promotes healthy skin and mucous membranes; possible role in preventing cataracts.
Niacin (B_3)	Energy production from foods; helps digestion, promotes normal appetite; promotes healthy skin.
Pantothenic acid (B_5)	Involved in energy production; helps formation of hormones
Pyridoxine (B_6)	Helps protein metabolism and absorption; helps in red blood cell formation.
Folate (B_9)	Helps in protein metabolism; promotes red blood cell formation; prevents birth defects of spine, brain; lowers homocysteine levels and thus coronary heart disease risk.

The vitamin content of leafy vegetables is one of the dietary advantages that contribute to the quality of minimally processed foods. However, it is also recognized that the vitamin content of the leaves can be easily lost during processing and storage. The main factors contributing to vitamin losses are oxidation, high temperatures, catalytic effect of metals (especially iron and copper), pH, action of degradative enzymes, moisture, irradiation (light or ionizing radiation), and various combinations of these factors. Each factor affects the vitamin stability in a different extent, being some vitamins more sensitive to processing and storage than others (65). The WSVs are more prone to leaching during washing, blanching, and domestic cooking. Thiamin (B_1) is heat-sensitive when the food pH is higher than 7, and unstable in air. Riboflavin (B_2) is vulnerable to decomposition by light, while niacin (B_3) and pyridoxine (B_6) are more resistant to processing conditions (65). In the other hand, vitamin C is very susceptible to chemical oxidation during processing, storage, and cooking. Regarding FSV stability, both vitamins

A and E can be destroyed under conditions that accelerate the oxidation of unsaturated fats, such as access of air, heat, light, trace metal ions, and storage time. Vitamin K is stable to heat, but extremely sensitive to both fluorescent light and sunlight. Vitamin D is little affected by processing and storage (66).

Vitamin C is normally one of the principal vitamins found in leafy vegetables, but it is also one of the most unstable. It is also one of the most studied vitamin in green leafy vegetables, including baby leaf products (20, 24, 28-30, 39, 41, 67). The initial content of ascorbic acid can be influenced by sunlight exposure during growth. In most green leafy vegetables a positive correlation between light and ascorbic acid content was found (24, 39, 44, 67). Vitamin C can occur as ascorbic acid and as its first oxidation product, the dehydroascorbic acid. The proportions of both species tend to vary with storage time, owing to the time-dependent oxidation of ascorbic acid. The rate of this reaction is affected by storage conditions and processing. During storage, the dehydroascorbic acid can be also irreversibly degraded, losing their biological activity. This reaction is potentiated by the presence of oxygen, metal ions, heat, high pH values and oxidases (28, 44, 66).

Vitamins evaluation is important for quality assurance purposes, regulatory compliance, and for establishing nutrient intakes of different populations (64). However, measurement of vitamins in foods is difficult due to the high variability in their composition, amounts and the great number of components in food matrices that interfere in vitamin analyses. Additional problems come from the existence of several structurally related chemical compounds with vitamin activity (vitamers). These have, in most cases, similar qualitative biological properties to one another, but, due to slight differences in their chemical structures, may exhibit different potency. Also provitamins (vitamin precursors, converted into vitamins by normal body metabolism) and the different factors that affects the vitamin stability contribute to the difficulty in assessing the food vitamin content (64-66). Traditionally, methods for vitamin determination require the analysis of each vitamin individually by widely differing physical, chemical and biological methods. The development of new analytical techniques has led, in recent years, to the development of analytical methods that are more rapid, accurate, and sensitive. Different chromatographic approaches have shown to be a good option in the determination of vitamin in food matrices (64, 65), especially, for the simultaneous determination of several naturally occurring vitamins (68-70).

The development of an analytical method to the determination of a several free WSV and FSV in green-leafy fresh-cut vegetables was one of the objectives of this thesis

and this subject is further discussed in chapter 3. Also the effect of the storage on the vitamin content of fresh-cut product will be addressed.

2.4.3.1.3 Minerals

Minerals are essential protective micronutrients needed for the maintenance of essential physicochemical processes, having a vital role in metabolic functions, normal growth and development (71). They are involved in the maintenance of pH, osmotic pressure, nerve conductance, muscle contraction, energy production and other metabolic reactions (72). Minerals may be broadly classified as macro (major) or micro (trace) elements. The macro-minerals (K, Na, Ca, Mg and P) are essential for human beings in amounts above 50 mg/day, while micro-minerals (Fe, Zn, Cu, Mn, I, F, Se, Cr, Mo, Co and Ni) are essential in concentrations below 50 mg/day (73). Regarding the recommended dietary intake of minerals, the green leafy vegetables can represent an importance source, being able to provide for human metabolism requirements. In plants the concentration of P, K, Ca and Mg can range from 1 to 15 mg/g dry weight (d.w). Potassium is the most abundant individual mineral in vegetables, and has a major role in cellular growth and stomatal function, balancing the charges of anions, activating almost 60 plant enzymes and participating in numerous metabolic processes, including protein synthesis, oxidative metabolism and photosynthesis. In a much lower concentration (minus 100- to 10 000-fold), can be found the following minerals Mn, Cu, Fe, Zn, Co, Na, Cl, I, F, S, and Se (44).

The minerals present in vegetable products can have a direct and an indirect effect on human health. The direct effects of minerals are those that come from the action of the minerals in the human metabolism (44). In table 2.3 are presented some of the effects of the most present minerals in leafy vegetables. The indirect effects result from the action of the minerals on the visual quality of the vegetables that will influence the consumer acceptance. Some minerals can affect the colour. Magnesium is present in the chlorophyll molecule, and is essential to maintain chlorophylls structure and consequently the green colour of the vegetables. A good correlation between iron supply and chlorophyll content was also found in vegetables, where an inadequate supply of this element can lead to an abnormal chlorophyll development and formation of prominent green veins in younger leaves that diminish the product quality (44).

Table 2.3 Major functions of the minerals found in leafy vegetables (completed with information from Otten et al. (73)).

Mineral	Proposed Effects on Human Metabolism
Potassium (K)	Main intracellular cation in the body, required for normal cellular function; the ratio of extracellular to intracellular K levels affects neural transmission, muscle contraction, and vascular tone; maintain lower blood pressure levels; decrease bone loss.
Sodium (Na)	Necessary to maintain extracellular fluid volume and plasma osmolality. High intake has severe adverse effects related to high blood pressure, which is a risk factor for heart disease, stroke, and kidney disease
Calcium (Ca)	Necessary for bone and teeth health (99% of body calcium); the remainder is involved in vascular contraction and vasodilation, muscle contraction, neural transmission, and glandular secretion.
Magnesium (Mg)	Involved in more than 300 enzymatic metabolic processes; maintenance of bone health and intracellular levels of K and Ca.
Phosphurous (P)	Major component of bones and teeth; helps maintain a normal pH in the body; and is involved in several metabolic processes.
Iron (Fe)	Critical component of several proteins, including enzymes, cytochromes, myoglobin and hemoglobin.
Zinc (Zn)	Component of various enzymes; maintenance of the structural integrity of proteins; regulation of gene expression.
Manganese (Mn)	Formation of bone and in specific reactions related to amino acid, cholesterol, and carbohydrate metabolism.
Cupper (Cu)	Component of several metalloenzymes, which act as oxidases in the reduction of molecular oxygen.

The presence of Ca in vegetables was associated with the maintenance of rheological properties of the cell wall. The cation Ca^{2+} interacts with the anionic pectic polysaccharides, coordinating with the oxygen functions of two adjacent pectin chains that form the cross linking, essential to the structure of vegetable tissues. Postharvest calcium treatments of fresh-cut products have been reported to retain firmness in different horticultural products. The effect of calcium was attributed to calcium's ability to cross-link with the pectic polysaccharide network, reducing the accessibility of cell wall enzymes to their substrates (25).

The mineral content found in vegetables is influenced by several internal and external factors. Genetics is determinant for mineral content, with plants accumulating higher concentrations of nutrients that are less mobile within the plant tissues (44). Pre-harvest factors like soil fertility (affected by pH, availability of nutrients and moisture), growth temperature and cultural practices (fertilization and irrigation) affect the mineral content of the plant. Also the maturity of the plant at harvest can influence the final mineral content in these products (74).

The determination of mineral content can be performed by several analytical methods. An overall mineral content can be determined by ashing, while atomic absorption spectrometry (AAS) or inductively coupled plasma spectrometry (ICP) techniques are applied to the determination of individual elements (44, 72).

The mineral composition of different baby leaf vegetables was an objective of this thesis, and this subject is further discussed on chapter 4. Besides the effect of maturity stage, also the influence of storage was evaluated.

2.4.3.2 Phytonutrients

Plants produce a very diverse set of organic molecules, whose function is not directly related to the major processes involved in growth and development (3). In general, these phytochemicals are secondary plant metabolites, synthesised by plant cells for functions beyond the primary needs of the cell, contributing to the survival of the whole plant as a functional organism. Some phytochemicals confer colour or odour, others act as signalling molecules, within the plant itself or in interactions with other organisms, and many are believed to function as natural pesticides (75). The plant secondary metabolites can be divided into four major groups: terpenoids (about 25000 compounds), alkaloids (about 12000 compounds), phenolic compounds (about 8000 compounds), and sulphur containing compounds, and are often unevenly distributed among the plant taxonomic groups (3). Some of those phytochemicals are biologically active organic substances, beneficial for human health, but without a specific human deficiency disorder identified (75). In this thesis, these compounds are referred as “phytonutrients”. A special attention will be given to the phenolic and carotenoid compounds, once they represent the major classes of phytonutrients in the majority of fresh-cut leafy vegetable. These compounds are recognized for their *in vitro* antioxidant capacity, leading to the assumption that this is their most important role in the human metabolism (75).

2.4.3.2.1 Phenolic Compounds

"Phenolic compounds" is a generic term that refers to a large number of compounds widely dispersed throughout the plant kingdom and characterized by having, at least, one aromatic ring with one or more hydroxyl groups attached. Phenolics are produced via the shikimic acid pathway and the phenylalanine ammonialyase (PAL) is the key enzyme catalyzing the biosynthesis of phenolics from the aromatic amino acid phenylalanine and tyrosine (44, 76, 77). A crucial step in this biosynthetic route is the introduction of one or more hydroxyl groups into the phenyl ring. As result, these compounds are derived from the same phenylpropanoid unit (C_6-C_3) and this pathway produces a heterogeneous group of compounds, including the simplest benzoic acids (C_6– C_1) and cinnamic acids (C_6–C_3) and the more complex compounds such as flavonoids (C_6-C_3-C_6), proanthocyanidins ((C_6-C_3-C_6)^n), coumarins (C_6-C_3), stilbenes (C_6-C_2-C_6), lignans (C_6-C_3-C3-C_6) and lignins ((C_6-C_3)^n) (78). The most common benzoic acid derivatives are *p*-hydroxybenzoic, vanillic, syringic and gallic acid, while common cinnamic acid derivatives include p-coumaric, caffeic, ferulic and sinapic acids. The derivatives differ in the degree of hydroxylation and methoxylation of the aromatic ring (44). Flavonoids are the most numerous phenolic group and include several classes of compounds: the flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones and others compounds. In nature, the phenolics aglycones are usually found conjugated to sugars, organic acids and functional derivatives such as esters or methyl esters (76, 79).

In plants, the phenolic compounds have multiple roles, essential to the plant survival. They are part of the natural defense system against insects, fungi, viruses and bacteria and they can act as plant hormone controllers. In particular, flavonoids are involved in processes like UV protection, pigmentation, stimulation of nitrogen-fixing nodules and disease resistance. The anthocyanins, which correspond to sugar-conjugated forms of anthocyanidins, are responsible for red, blue and purple colours, having an important role in attracting insects for seed dispersion and pollination (76).

The different phenolic compounds contribute to visual appearance, flavour and nutritive quality of vegetables. Anthocyanins and other coloured flavonoids contribute to the plant colour (see section 2.4.1). The intensity of the colour is influenced by the pH and the presence of other co-pigments in the cell. Browning is also the result of two degradative enzymes over phenolic subtract (normally, monohydroxyphenols and orthodihydroxyphenols). The polyphenol oxidases (PPO) and peroxidases (POD) generate brown polymers (melanins) that decrease rapidly the quality of fresh-cut vegetables during shelf life (77). Regarding the role of phenolic compounds on vegetable flavour, there are several compounds contributing to the bitter, sweet, pungent and

astringent taste. Normally, low-molecular-weight phenolics tend to be bitter and higher-molecular-weight polymers are more likely to be astringent (56). Compounds like the flavone neohesperidosides, oleuropein and coumarins are responsible for the bitter sensation, while the presence of neohesperidin dihydrochalcone is an intense sweetener. The pungent sensation, characteristic of some spices, is attributed to the presence of capsaicins and curcuminoids, and the astringency to the presence of tannins, like ellagitannins and gallotannins (77). Molecules that have one dihydroxyphenyl moiety, like chlorogenic acid, catechin and epicatechin can also cause some astringency sensation (77).

The nutritional quality of fresh-cut vegetables is closely related to the presence of antioxidant compounds, specially, to their phenolic content. These have been intensively investigated due to their potential health-promoting effects (76, 77). Besides their antioxidant activity (the phenolics can retard or inhibit lipid autoxidation by acting as radical scavengers), some phenolic compounds have shown to possess anti-inflammatory, enzyme inhibition, antimicrobial, antiallergic, vascular and cytotoxic antitumor activity (3, 75, 76, 79).

Several factors determine the accumulation and degradation of phenolic compounds in plants, affecting subsequently the nutritional value. Once more, genetic and environmental factors during growth, at harvest and during shelf life are determinant for the phenolic compounds accumulation and stability (44). Genetics (species and variety) determines the prevalence of different phenolic compounds (76). Regarding the effect of the environment, different biotic stresses (insect attack and pathogen infection) and abiotic stresses (light, temperature, nutrient supplies, water availability, growing conditions and UV radiation), the maturity at harvest, the storage conditions and the processing methods have influence on the final concentration of polyphenols in plant tissues (44, 76). Fresh-cut vegetables are often associated to an increased phenolic content, generated in response to wounding caused by minimal processing (cutting, handling) or use of modified atmosphere packaging (80). For example, in the phenylpropanoid pathway, the phenylalanine ammonialyase (PAL) is activated by wounding, generating more phenolic compounds in certain fresh-cut products (77, 81). On the other hand, some studies had reported a decrease of the phenolic content during shelf life of fresh-cut products. The behaviour of phenolic content (decrease, maintain or increase) during shelf life seems to be dependent on the type of product, on the minimal processing methods, on the temperature and duration of the storage (76, 80). The wound response also seems to be influenced by the initial levels of other compounds, like reduced ascorbic acid and phenolic compounds (82).

The content and the stability of the phenolic compounds in fresh-cut baby leaf vegetables is determinant for the nutritional quality of these products. The phenolic profile of some baby leaves, like spinach, lamb´s lettuce, lettuce, mizuna, wild rocket and watercress was described by different authors (14, 28, 29, 34, 42). The majority of the works have focused on the phenolic content, comparing in some cases, the baby leaf phenolic content with more mature leaves (14). Baby sized leaves of lettuce and spinach showed higher content of phenolic compounds compared to the more mature leaves, being the phenolic content highly determined by the genotype of the product (14, 28).

The identification and quantification of phenolic compounds can be achieved by spectrophotometric methods (eg. Folin–Ciocalteu assay for the determination of the total soluble phenolic content) or with chromatographic techniques (LC or GC) combined with diode array (DAD) and/or mass detection (78). The determination of the phenolic profile of different baby leaf vegetables, and also the study of their stability during storage was an objective of this thesis, being these subject further discussed on chapter 5.

2.4.3.2.2 Carotenoids

Carotenoids are terpenoid compounds that contain forty carbon atoms, ubiquitous in plants, mainly as components of chloroplasts. They occur as pigments and are associated with the photosynthetic apparatus. There are two main classes of carotenoids: the carotenes (without oxygen atoms); and the xanthophylls (with oxygen atoms). In nature, they exist primarily in all-*trans* isomeric form (more stable), but *cis* isomers also occur in a lesser extent. The figure 2.6 shows a scheme of the carotenoid compounds formation in nearly all plant species (83). All steps are mediated by different biosynthetic enzymes. The first step of carotenoid biosynthesis is the condensation of two molecules of GGPP to form the first C40 carotenoid, a phytoene. The carotenoid pathway branches at the cyclization reactions of lycopene to produce carotenoids with either two β-rings (e.g. β-carotene, zeaxanthin, antheraxanthin, violaxanthin and neoxanthin) or carotenoids with one β-ring and one δ-ring (e.g. α-carotene and lutein). The pathway advances with the additions of oxygen moieties, which convert the hydrocarbon α- and β-carotenes into xanthophylls. Further steps in xanthophyll synthesis include epoxydation reactions. The reversible epoxidation/ de-epoxidation reaction converting violaxanthin back to zeaxanthin via the intermediate antheraxanthin (known as violaxanthin cycle) is crucial for energy dissipation from incoming solar radiation (85). In some vegetables, a modified carotenoid biosynthetic capacity produce compounds exclusively associated with their respective genus, or even species, like the formation of lactucaxanthin in lettuce species (85).

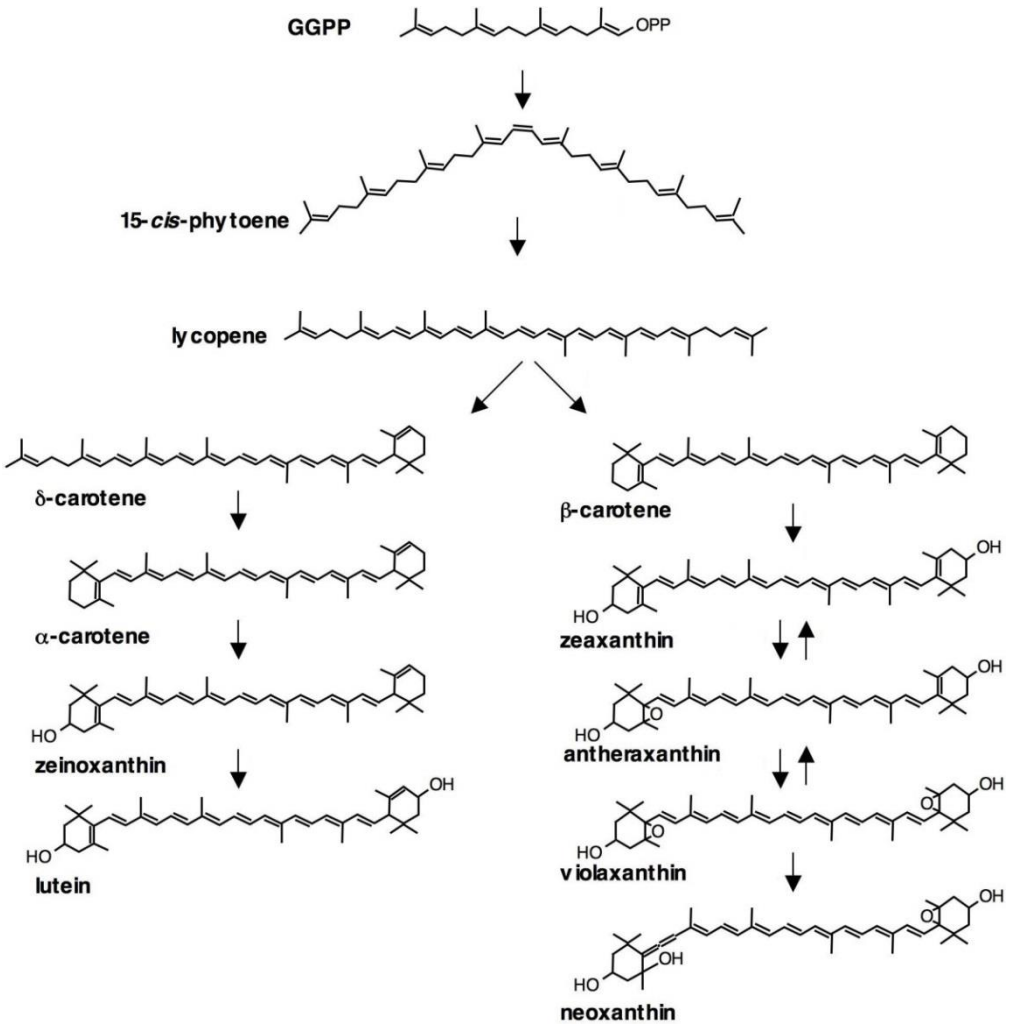

Figure 2.6 Carotenoid biosynthetic pathway in land plants (GGPP: geranylgeranyl pyrophosphate) (adapted from DellaPenna and Pogson (84)).

The most commonly occurring carotenes are β-carotene in vegetable chloroplasts' and lycopene in chromoplasts of some flowers and fruits. The most abundant xanthophylls in photosynthetic plant tissues, lutein, violaxanthin, and neoxanthin, are key components of the light-harvesting complexes. Carotenoids function in plants is to help harvesting light energy, mostly in the blue–green wavelength range, which is transferred to the photosynthetic reaction centres (RCs). In the PSII complex, β-carotene is highly concentrated close to the reaction centre, and lutein is present in several light harvesting antennae components. Besides this function, carotenoids are also involved in photoprotection, photomorphogenesis, nonphotochemical quenching and lipid peroxidation mechanisms (85). The carotenoid composition of leafy vegetables follows, in general, the same pattern. Lutein is the predominant carotenoid in green leaves and green vegetables (about 45%), followed by β-carotene (usually 25–30%), violaxanthin (15%), and neoxanthin (15%). The absolute concentrations can vary significantly (83).

From the more than 600 known carotenoids in nature, only a few have recognized nutritional significance for humans. The provitamin A carotenoids (β-carotene, α-carotene, δ-carotene and β-cryptoxanthin) are important precursors of vitamin A, being converted in the human intestinal mucosa. β -carotene and other carotenoids are potent antioxidants, and certain compounds, including the xanthophylls lutein and zeaxanthin, can accumulate in the macula lutea of the human eye and the corpus luteum of the ovaries, where they have an important protective role against free-radical mediated damage (75). The ingestion of food rich in carotenoids (fruits and vegetables) was associated with a low incidence of cardiovascular disease. Carotenoids can inhibit cell proliferation, cell transformation, and modulate the expression of gene determinants in the prevention of certain types of cancers (85).

Carotenoid accumulation in plants appears to be dependent on genetic information (species and variety), as well as on environmental growth factors such as light, temperature and fertility. Also stage of maturity, harvesting, postharvest handling and storage can influence the carotenoid composition (83, 85). In baby leaf samples the carotenoid content is expected to be highly influenced by their maturity stage. In a study about the carotenoid content of brassica specie (kale) in different stages of maturity, the fully mature leaves had higher carotenoid amounts than baby sized leaves, with senescent leaves having the lowest carotenoid levels. The pigment accumulation on these leaves reached a maximum between the 1st and 3rd week of leaf age, decreasing during senescence phase (86).

The carotenoids carbon chain reacts with light, acids, heat, and oxygen. As a consequence, carotenoids may lose colour and also their biological activity. Moreover, naturally occurring *trans* isomers carotenoids are susceptible to *cis*−*trans* isomerization reactions during food processing, producing colourless compounds that might affect the product flavour and visual quality (87).

The analysis of carotenoids is not easy due to the diversity and the presence of *cis*–*trans* isomeric forms of this group of compounds. The reaction with light, acids, heat, and oxygen is another drawback in the analysis of carotenoid compounds, making them unstable during handling. In this analysis the use of antioxidant compounds, such as BHT and protection from light is recommended (83, 88). As lipid pigment compounds, the extraction of carotenoids from the vegetable matrix is normally performed with aprotic and low polarity organic solvents (88). HPLC with DAD and MS detection is currently the chosen method for carotenoid analysis Due to the difficulty of the carotenoid identification based only on their UV–Vis spectra (there are several carotenoids with a very similar UV–

Vis spectra), the information provided by MS detector is necessary to differentiate compounds with diverse molecular mass. However, in the case of isomers or carotenoids with the same chemical composition, it would be necessary to conjugate both, the information provided by DAD and MS analyses (88). The identification and quantification of carotenoid compounds from a baby leaf sample was performed during the development of this thesis. Thus, this subject will be further discussed on chapter 6.

2.4.3.3 Antioxidant Properties

The vegetables phytochemicals, especially some vitamins, phenolics and carotenoids, are believed to have an important role in preventing diseases caused by oxidative stress. Oxidative stress reactions release free oxygen radicals, which are related with the onset of numerous disorders including cardiovascular malfunction, cataracts, cancers, rheumatism and other auto-immune diseases (89). Free radicals are highly reactive, short-lived species generated in normal oxidative metabolism reactions and by a several biological mechanisms, like inflammation process. Reactive oxygen species (ROS) correspond to reduced forms of oxygen such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$) or hydroxyl radical ($OH^{\bullet}$). ROS are natural by–products of the normal metabolism of oxygen and have important roles in cell signalling and homeostasis. However, in case of imbalance caused by stress factors, the ROS levels can increase significantly originating oxidative stress (90). Besides ROS, also some molecules with unpaired electrons are able to cause oxidative damage (44). ROS can readily interact with lipids, proteins and nucleic acids, damaging their structures and normal function and leading, eventually, to cell death. To counteract the damages caused, cells have different antioxidant mechanisms (75). From a biological perspective, an antioxidant is any compound able to oppose cellular oxidation. According to its nature, they may be classified as enzymatic (eg. superoxide dismutase, catalase, glutathione) or non– enzymatic antioxidant compounds (eg. vitamins, phenolics and many others).

Diets rich in fruits and vegetables have been associated with reduced incidence of diseases caused by oxidative damage, being the protective effects associated to their content of different phytochemicals such as natural antioxidants, fibres and other bioactive compounds that may be beneficial for human metabolism (89).

Within the most investigated dietary compounds in vegetables with a role in antioxidant defences are vitamins A, C, and E, different phenolic compounds, carotenoids, chlorophylls and others (see Figure 2.7). These compounds may act independently or in combination as protective agents by a variety of mechanisms (89).

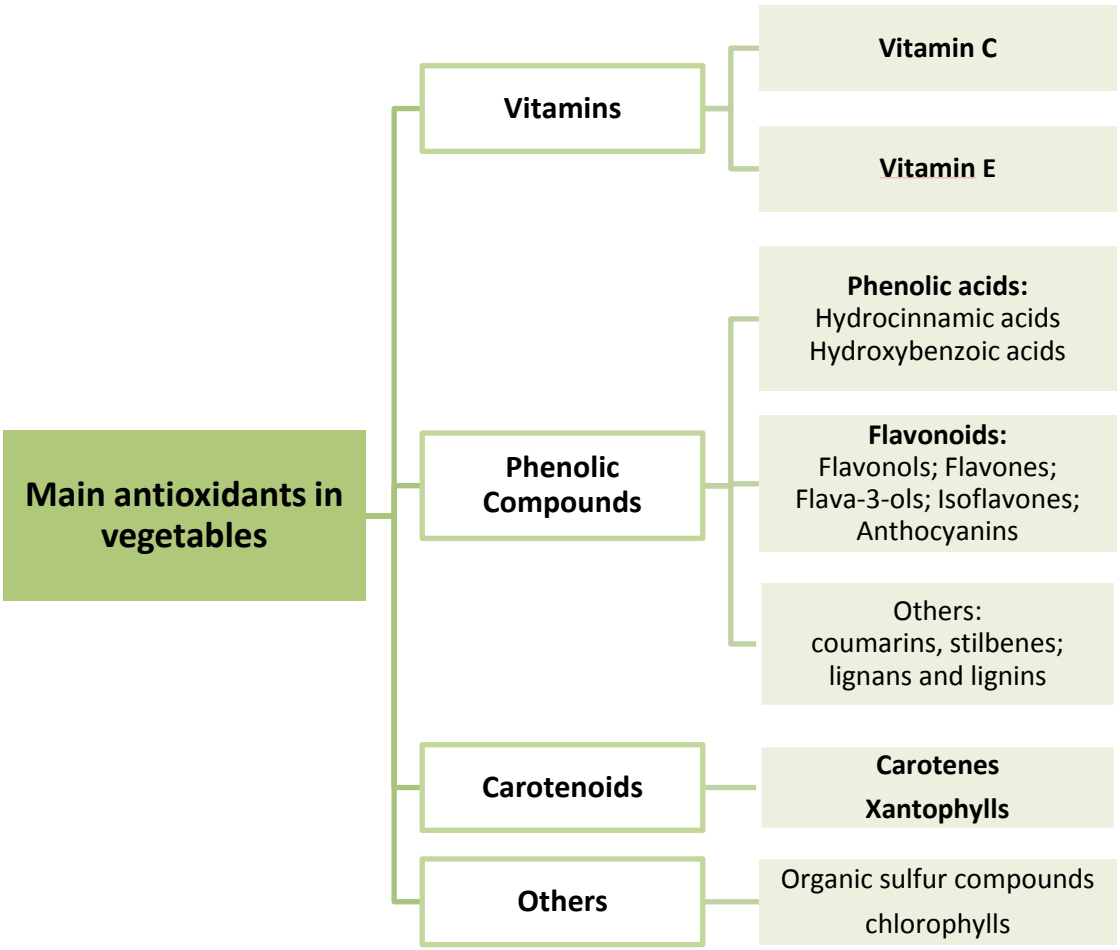

Figure 2.7 Main dietary antioxidants present in vegetables (adapted from Vicente et al. (44)).

Vitamin C is one of the most important compounds for human nutrition present in fruits and vegetables for its high capacity to neutralize ROS. This vitamin is a powerful electron donor, reducing ROS to water, and generating an oxidized form of ascorbate that is relatively stable and unreactive, and do not cause cellular damage (75, 90). Vitamin C also is involved in the regeneration α-tocopherol from α-tocopherol radicals in membranes and lipoproteins (90). Vitamin E (α-tocopherol) is an important lipid-soluble antioxidant nutrient that normally accumulates in cell membranes, and acts by reacting with lipid free-radicals, blocking peroxidation chain reactions and protecting cell membranes from oxidative damage (75). Phenolic compounds are the antioxidant compounds normally associated to the higher antioxidant defences provided by vegetables and fruits (75). These compounds can inactivate ROS by several mechanisms. The phenolic hydroxyl groups are good hydrogen donors to ROS, working as terminators of free radical chains and as chelators of redox-active metal ions that are capable of catalyzing lipid

peroxidation (90). Another mechanism of action is related to the phenolic potential to interact with proteins, which make them capable of inhibit some enzymes involved in radical generation, such as various cytochrome P450 isoforms, lipoxygenases, cyclooxygenase and xanthine oxidase. Moreover, several synergistic effects of phenolics with other antioxidant compounds, namely ascorbic acid, β-carotene and α-tocopherol, and the regulation of intracellular glutathione levels have been described (78). Regarding the antioxidant capacity of carotenoid compounds, the physical properties of the molecules, in particular the conjugated carbon–carbon double bond system, permits to delocalize unpaired electrons to inactivate ROS. This is primarily responsible for the excellent ability of α-carotene to physically quench singlet oxygen without degradation (85, 90).

Besides the molecular structure of each compound, the antioxidant capacity also depends on their concentration on the tissues as well as on the ROS nature and concentration. The total antioxidant capacity also results from synergistic interactions between different antioxidant compounds. The concentration of each compound in the vegetables is affected by several factors, as it was stated in previous sections of this chapter. By its turn, the antioxidant capacity of the vegetables is dependent on the several physiological mechanisms and on the impact of processing and storage (75, 85).

In baby leaf products, the more immature leaves have normally higher respiration rates (30), which may have a negative impact in the stability of the different antioxidant compounds. However, the opposite situation was described in baby leaf samples of spinach, where the higher content of vitamin C in younger leaves increased the baby leaves ability to resist to oxidative stress during storage, leading to a lower rate of senescence (28).

The nutritional quality of fresh-cut vegetables is closely related to the presence of antioxidant compounds. The evaluation of these compounds is, therefore, an indicative of antioxidant capacity of these products. Presently, there are also many methods used to determine total antioxidant activity, but all of them with some limitations. Different antioxidant assay methods have given results that showed different trends, being necessary to use more than one method to study the antioxidant capacity of a product. Among several antioxidant assay available, the most used include the Trolox equivalent antioxidant capacity (TEAC) assay, the oxygen radical absorbance capacity (ORAC) assay, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and ferric ion reducing antioxidant parameter (FRAP) assay (89). The measure of the total antioxidant capacity was used in the development of the thesis experimental work, as a part of the evaluation of the

nutritional quality of fresh-cut vegetables, being this subject further discussed on chapter 7.

2.5 Summary

The fresh-cut baby leaf products are appellative and convenient for both producers and consumers. However, the maturity stage, key concept of this product, influences the final quality of the leaves. With the different minimal processing techniques, and leaves being packed whole, the visual appearance, flavour and texture seems to attract consumers and be adequate to the fresh-cut industry. Due to the use of gentle handling practices, the smaller cut surface (normally at the base of the leaf) and a strict control of the storage temperature, the leaves are able to maintain their fresh appearance (green colour without browning spots due to phenolic oxidation at the cut surface) during their shelf life period. Regarding the nutritional quality of this leaves, the higher metabolism rate of more immature leaves can lead to a different evolution of the phytonutrient content during storage, comparing to the more mature fresh-cut leafy vegetables. These differences are greatly dependent on the specie and can be reflected, mainly, on the total antioxidant capacity of these new products. Vitamins, minerals, phenolics and carotenoids are the main phytochemicals that represent, together with their high dietary fibre content, the main nutritional advantages of vegetables. The behaviour of these compounds during shelf life will be determinant to the nutritional quality of baby leaf products and consequently for the continuous success of these products. Consumers give now more importance to the nutritional value, seen as a possibility to preserve and improve their health through diet, choosing more consciously products rich in antioxidants compounds.

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CHAPTER 3.

Sequential determination of fat- and water-soluble vitamins in green leafy vegetables during storage

This chapter presents a new method to sequentially analyse free forms of fat- and water-soluble vitamins in different green leafy vegetables, using ultrasound extraction and LC–MS/MS and LC–DAD. The content of each vitamin was assessed before and after storage.

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Sequential determination of fat- and water-soluble vitamins in green leafy vegetables during storage

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Abstract

The simultaneous analysis of fat- and water-soluble vitamins from foods is a difficult task considering the wide range of chemical structures involved. In this work, a new procedure based on a sequential extraction and analysis of both types of vitamins is presented. The procedure couples several simple extraction steps to LC-MS/MS and LC-DAD in order to quantify the free vitamins contents in fresh-cut vegetables before and after a 10-days storage period. The developed method allows the correct quantification of vitamins C, B1, B2, B3, B5, B6, B9, E and provitamin A in ready-to-eat green leafy vegetable products including green lettuce, ruby red lettuce, watercress, swiss chard, lamb's lettuce, spearmint, spinach, wild rocket, pea leaves, mizuna, garden cress and red mustard. Using this optimized methodology, low LOQs were attained for the analyzed vitamins in less than 100 min, including extraction and vitamin analysis using 2 optimized procedures; good repeatability and linearity was achieved for all vitamins studied, while recoveries ranged from 83% to 105%. The most abundant free vitamins found in leafy vegetable products were vitamin C, provitamin A and vitamin E. The richest sample on vitamin C and provitamin A was pea leaves (154 mg/g fresh weight and 14.4 mg/100 g fresh weight, respectively), whereas lamb's lettuce was the vegetable with the highest content on vitamin E (3.1 mg/100 g fresh weight). Generally, some losses of vitamins were detected after storage, although the behavior of each vitamin varied strongly among samples.

Keywords: fresh-cut vegetables, fat-soluble vitamins, water-soluble vitamins, LC-MS/MS, green leafy vegetables.

3.1 Introduction

Vitamins are biologically active organic compounds that are essential micronutrients involved in metabolic and physiological functions in the human body. There are thirteen vitamins identified that are classified according to their solubility into fat-soluble vitamins (FSV) (A, E, D, and K) and water-soluble vitamins (WSV) (B-group vitamins and vitamin C) [1]. These compounds greatly differ in their chemical composition, physiological action and nutritional importance in the human diet, even within the same group [2]. The FSV are involved in complex metabolic reactions related to important biological functions, such as vision (vitamin A), calcium absorption (vitamin D), antioxidant protection of cell membranes (vitamin E) and blood coagulation (vitamin K), among other functions [3]. Several vitamins of the B-group act mainly as coenzymes in the catabolism of foodstuffs to produce energy [1].

WSV and FSV are one of the micronutrients that are usually labeled in foods. In this sense, minimally processed vegetables (e.g. lettuce, wild rocket, watercress, spinach) are not an exception. These products are basically ready-to-eat foods composed by raw vegetables that retain as much of the naturally occurring vitamin content. However, there are several factors that can lead to vitamin losses in these products such as temperature, the presence of oxygen, light, moisture content, water activity, pH, enzymatic modifications and metal trace elements, particularly iron and copper [1].

The degree of degradation will vary according to the vitamin and could also be affected by the processing and storage time to which the vegetable is submitted. It is known that WSV are more susceptible to leaching losses during washing, while vitamin C is very prone to chemical oxidation during processing and storage stages [1]. Vitamins A and E could be destroyed under the presence of oxygen, light, heat, trace metal ions and storage time [1]. Therefore, monitoring the vitamin content during processing and storage is of great importance to food technologists and consumers to assure the nutritive value of foods, and also for quality assurance purposes and regulatory compliance. This requirement creates the need for more rapid and specific methods for vitamin determination [4-6].

The development of a single method for the multiple and simultaneous monitoring of WSV and FSV is very challenging due to different reasons. The level of vitamins in food may be as low as few micrograms per 100 g, usually very unstable and accompanied by an excess of compounds with similar chemical behaviour. Traditionally, methods for vitamin determination require the analysis of each vitamin individually by using different

physical, chemical and biological methods. Microbiological assays are still the methods of reference as Official Methods of Analysis of AOAC International for some vitamins (vitamin B_5, B_6, B_9 and B_{12}); these are highly sensitive but also laborious to achieve an estimation of mean value with a certain precision [1,6,7]. High performance liquid chromatographic (HPLC) methods are often used for the determination of WSV and FSV. The choice of the method depends on the accuracy and sensitivity required, as well on the interferences encountered in the sample matrix. HPLC, with UV absorbance and/or fluorescence detection is well established for both FSV and WSV measurements, but showed some limitations for certain analytes and also lacks specificity in complex matrices [8]. Liquid chromatography-mass spectrometry (LC-MS) shows more sensitivity and specificity for the determination of vitamins in these matrices, and permits the simultaneous analysis of multiple vitamins in a single analysis [8,9]. The majority of the HPLC multivitamin methods found in the literature focused only either FSV or WSV and were mainly applied to analysis of pharmaceutical preparations or supplemented foods [5,10-13]. Only some of them attempted the determination of naturally occurring vitamins on food [8,9,14-18]. Moreover, to the best of our knowledge, the determination of a wide group of free vitamins in green-leafy fresh-cut vegetables has not been carried out.

Consequently, the objective of the present work is to develop and validate a HPLC-DAD-MS/MS-based method that allows a simple and sequential extraction and monitoring of several free forms of WSV (vitamins C, B_1, B_2, B_3, B_5, B_6 and B_9) and FSV (pro-vitamin A and vitamin E) in raw green leafy vegetables to study their contents as well as their evolution along a typical storage period emulating the market conditions.

3.2 Materials and Methods

3.2.1 Samples

Twelve samples of green leafy vegetables from seven different families (Asteraceae, Brassicaceae, Chenopodiaceae, Valerianaceae, Alliaceae, Amaranthaceae, and Fabaceae) were obtained from a producer of minimally processed vegetables (Odemira, Portugal). The samples used were fresh-cut leaves of red ruby lettuce and green lettuce (*Lactuca sativa var. crispa*), watercress (*Nasturtium officinale*), swiss chard (*Beta vulgaris*), lamb's lettuce (*Valerianella locusta*), spearmint (*Mentha spicata*), spinach (*Spinacia oleracea*), wild rocket (*Diplotaxis muralis*), pea (*Pisum sativum*), mizuna (*Brassica rapa* var. *japonica*), garden cress (*Lepidium sativum*) and red mustard (*Brassica juncea*). The samples were freeze-dried (Telstar Cryodos-80, Terrassa, Barcelona) upon

arrival and after 10 days of refrigerated storage (3 ± 1 ºC). The freeze-dried leaves were reduced to a fine powder in a knife mill (GM 200, RETSCH, Haan, Germany) and stored protected from light, oxygen and heat until analysis. The freeze dried samples were spiked with vitamins standards in order to identify and quantify these vitamins forms in the real samples.

3.2.2 Chemicals and standard solutions

All chemicals used were of analytical reagent grade. Vitamins standards (purity >99.0%), namely, ascorbic acid (C), thiamine hydrochloride (B_1), riboflavin (B_2), nicotinamide (B_3), D-calcium pantothenate (B_5), pyridoxine (B_6), folic acid (B_9), α-tocopherol (E) and β-carotene (provitamin A), were purchased from Sigma Aldrich (Madrid, Spain). The internal standards, hippuric acid and trans-β-Apo-8′-carotenal as well as triethylamine (TEA) and butylated hydroxytoluene (BHT) were also from Sigma Aldrich (Madrid, Spain). Ammonium acetate and acetic acid were from Panreac (Barcelona, Spain) and Scharlau (Sentmenat, Spain), respectively. Methanol (MeOH), methyl *tert*-butyl ether (MTBE) and ethyl acetate were HPLC-grade from Lab-Scan (Gliwice, Sowinskiego, Poland). Distilled water was deionized by using a Milli-Q system (Millipore, Bedford, MA, USA).

Individual WSVs standard solutions and hippuric acid (1mg/ml) solution were prepared in 10 mM ammonium acetate (pH 4.5), and kept in the dark under refrigeration at 4 ºC until analysis. Ascorbic acid was prepared at 5mg/ml, thiamine hydrochloride, nicotinamide, D-calcium pantothenate and pyridoxine at 1 mg/ml, riboflavin at 0.05 mg/ml and folic acid at 0.01 mg/ml. During method development, a mixture of WSVs were prepared daily by dilution of the individual vitamins stock solutions with 10 mM ammonium acetate solution with concentrations within the range of the values reported by nutritional tables for the samples under study (C: 3.3 µg/ml; B_1: 0.6 µg /ml; B_2: 0.75 µg/ml; B_3: 1.75 µg/ml; B_5: 0.8 µg/ml; B_6: 0.67 µg/ml; B_9: 0.33 µg/ml). α-tocopherol, β-carotene and trans-β-Apo-8′-carotenal were dissolved in MeOH (1 mg/ml) and stored at -20 ºC, protected from light. A mixture of these fat soluble vitamins was also prepared before injection at 0.1 mg/ml with ethyl acetate.

3.2.3 Samples extraction

A scheme of the extraction procedure developed in the present work to simultaneously extract WSV and FSV is shown in Figure 3.1. During the extraction

process, samples were always protected from direct exposition to light and kept on ice to minimize vitamins degradation.

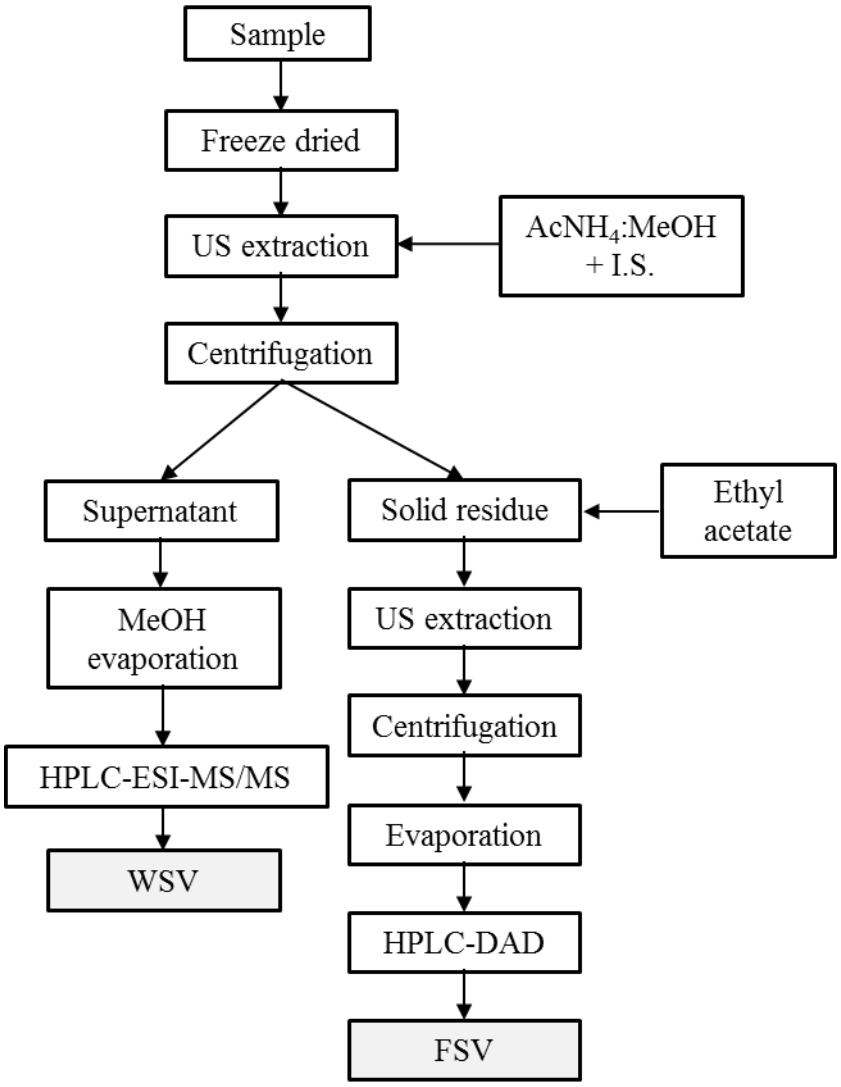

Figure 3.1 Extraction scheme for WSV and FSV. US, ultrasounds; $AcNH_4$, ammonium acetate.

Briefly, 0.250 g of each sample was first extracted with 16 ml of 10 mM ammonium acetate/methanol 50:50 (v/v) containing 0.1% BHT. Standard solutions (40 µl of 1mg/ml hippuric acid and 40 µl of 1mg/ml trans-β-Apo-8′-carotenal) were added at this stage. Hippuric acid concentration added was of 2.5 µg/ml and of 3.3 µg/ml for trans-β-Apo-8′-carotenal. After 15 minutes of shaking to achieve good sample dispersion in the extraction liquid, samples were placed in an ultrasound bath for 15 minutes. Bath temperature was always controlled with ice to guarantee that water temperature did not rise above 25 ºC. The samples were centrifuged at 14000 g for 15 min and the supernatant was withdrawn and filtered through a 0.45 µm nylon filter. One ml of the supernatant was concentrated into a nitrogen stream to evaporate the methanol and it was injected into a HPLC-MS/MS

system to determine the WSV content. The solid residue from the first extraction was re-extracted twice with ethyl acetate containing 0.1% BHT (6 + 6 ml) for 15 minutes, also in the ultrasonic bath. Finally, the samples were centrifuged (14000 g, 15 min) and the supernatants combined and filtered through a 0.45 µm nylon filter. The extract was taken to dryness under a N_2 stream. The residue was dissolved in 3 ml of ethyl acetate and injected in a HPLC-DAD system to monitor the pro-vitamin A (β-carotene) and vitamin E (α-tocopherol) contents.

3.2.4 Analysis of water soluble vitamins (HPLC-DAD-MS/MS)

HPLC–MS/MS analyses of WSV were performed using an Accela liquid chromatograph (Thermo Scientific, San Jose, CA) equipped with a diode array detector (DAD), an autosampler and a TSQ Quantum triple quadrupole analyzer (Thermo Scientific). The chromatograph was coupled to a MS analyzer via an electrospray (ESI) interface. Xcalibur software (Thermo Scientific) was used to analyze and store the data. The column used was an ACE-100 C_{18} (100 x 2.1 mm i.d., 3µm particle size) (Advanced Chromatographic Technologies, Aberdeen, UK).

The method developed to simultaneously separate the seven forms of WSV in a single run was based on the work of Vazquez et al. [19] with some modifications, using 10 mM ammonium acetate solution (pH 4.5) as mobile phase A, MeOH with 0.1% acetic acid as mobile phase B and MeOH with 0.3% acetic acid as mobile phase C. The gradient used is described on Table 3.1. The flow rate was 0.2 mL/min whereas the injection volume was 10 µL. The DAD recorded the spectra from 200 to 680 nm. Column and autosampler compartments were thermostated at 20 and 5 ºC, respectively. To identify and quantify the WSV, the mass spectrometer was operated first in the negative ESI mode, for 1.7 min. Spray voltage and capillary temperature were set at 3000 V and 250 ºC, respectively. These conditions were the most suitable for ascorbic acid detection. A second segment of 10.3 min followed, using positive ESI mode to monitor the presence of the other WSV. In this segment the spray voltage and capillary temperature were set at 5000 V and 250 ºC, respectively. Nitrogen was used as sheath and auxiliary gas at pressures of 40 and 19 a.u., respectively. Ion sweep gas pressure was 2 units and collision gas (Ar) pressure, 1.3 mTorr. Scan width and scan time were fixed at 0.020 (m/z) and 0.1 s, respectively, and the system was operated in selected reaction monitoring (SRM).

Table 3.1 Gradient Elution used on water soluble vitamins analysis.

Time (min)	A (%)	B (%)	C (%)
0	90	10	0
3	90	10	0
4	50	0	50
7	50	0	50
10	0	100	0
17	0	100	0
20	90	10	0
30	90	10	0

SRM parameters were optimized by direct injection of standards. Two ion transitions were monitored for identification but only the most intense product ion for each precursor ion was used for quantification. The values corresponding to the tube lens offset voltage and collision energy for each selected ion transitions are indicated in Table 3.2.

Table 3.2 Retention times (min) and MS/MS detection parameters for the water soluble vitamins analyzed.

Peak	Vitamin	t_r (min)	Precursor ion (m/z)	SRM transitions		Tube lens offset (V)
				Quantifier ion (m/z) (Collision energy, V)	Qualifier ion (m/z) (Collision energy, V)	
1	Ascorbic Acid (C)	1.41	174.9 $[M-H]^-$	115.2 (14)	87.3 (18)	76
2	Thiamine (B_1)	1.81	265.1 $[M+H]^+$	122.1 (10)	144.1 (16)	48
3	Pyridoxine (B_6)	2.30	169.9 $[M+H]^+$	152.1 (11)	134.1 (19)	55
4	Nicotinamide (B_3)	3.26	123.0 $[M+H]^+$	80.3 (16)	78.3 (24)	67
5	Pantothenic Acid (B_5)	3.72	220.0 $[M+H]^+$	202.1 (12)	184.1 (12)	60
6	Hippuric Acid (IS)	5.90	180.1 $[M+H]^+$	105.2 (10)	77.4 (10)	59
7	Folic Acid (B_9)	6.24	442.0 $[M+H]^+$	294.9 (13)	176.0 (34)	69
8	Riboflavin (B_2)	6.82	377.1 $[M+H]^+$	243.0 (23)	147.1 (37)	93

3.2.5 Analysis of fat soluble vitamins (HPLC-DAD)

The FSV determination was performed in an Agilent 1100 HPLC chromatograph (Agilent, Palo Alto, CA) equipped with an autosampler, a diode array detector (DAD) and an YMC C_{30} analytical column (5 µm particle size, 250 x 4.6 mm i.d.) (YMC, Schermbeck, Germany). The method used was based on the method previously published by Jaime et al. [20]. Methanol/water/TEA (90:10:0.1, v/v/v) and MTBE/methanol/water/TEA (90:6:4:0.1, v/v/v/v) were employed as mobile phases A and B, respectively. Elution was carried out using the following gradient: 0 min, 6.5 %B; 8 min, 6.5 %B; 43 min, 100 %B;

46 min, 6.5 %B; 55 min, 6.5 %B. The flow rate was 1 mL/min and the injection volume 10 µL. Chromatograms were monitored at 295 nm for α-tocopherol content and at 450 nm for carotenoid compounds.

3.2.6 Method validation

A recovery study for each vitamin was performed as follows using Spearmint as model sample: 2 sets of extractions were carried out simultaneously following the optimized procedure. In one of them, the sample spiked with a known amount of the target compounds was extracted whereas in the other, the same sample was extracted and spiked with the same concentration of vitamins after extraction. Peak areas of vitamins from the same sample spiked before and after the extraction process were compared. The recovery study was performed in three different days, always with the same sample in order to check its repeatability.

Once optimum separation conditions and MS/MS detection parameters were optimized, instrumental intraday and interday precision was assessed. For this purpose, a mixture of the studied vitamins was consecutively injected five times in the same day (n = 5) and also in three different days (n = 15). The vitamins concentration of this mixture was in the middle range of the linearity of each one (vitamin C: 1.32 µg/ml; B_1: 0.24 µg/ml; B_2: 0.3 µg/ml; B_3: 0.7 µg/ml; B_5: 0.33 µg/ml; B_6: 0.27 µg/ml; B_9: 0.13 µg/ml). Limits of detection (LOD) and quantification (LOQ) were calculated as a signal-to-noise ratio of 3 and 10, respectively and expressed in ng/ml. Accuracy of the method was assessed by using the recovery percentages of spiked samples.

3.2.7 Statistical analysis

IBM SPSS Statistics software v.19 was employed for data elaboration and statistical analysis using a level of significance set at 95 %. One-way analysis of variance (ANOVA) was employed to assess differences among vitamin contents at the different storage times. Differences were considered statistically significant if $p < 0.05$.

3.3 Results and Discussion

Two different approaches can be followed to determine several free forms of water- and fat-soluble vitamins in real samples. First one deals with the separation of all the vitamin forms in one single analysis and the second with the optimization of two individual

methods for a more accurate measurement of the different vitamin forms. Although several works have been published dealing with the simultaneous determination of several WSV and FSV in a single chromatographic run with DAD and MS detectors [15,19,21,22], none of them refer to the analysis in real complex food samples but instead analyze pharmaceutical preparations, functional drinks and parenteral nutrition admixtures. As mentioned previously, the main goal of the present work was to develop a useful and rapid method for the determination of both types of vitamins in real green leafy vegetables and to use it to follow vitamin evolution during cold storage; therefore, the method should be able to provide an accurate quantification of all the free vitamin forms and its changes. Main problems arisen in the determination of WSV and FSV in a single run using real complex samples deal with coelutions among the different vitamins and with other major components such as organic acids and polyphenols (that obviously are found in higher amounts), with the difficulty of selecting the best MS detection conditions, including the ionization technique, which are different for both groups of vitamins; etc. Considering all these aspects, our efforts were focused in the development of two different sequential methods that could provide good quantification capabilities and that could be employed in parallel. Therefore, optimization was carried out for the separation of WSV by HPLC-MS/MS and FSV by HPLC-DAD; and for the extraction protocol to recover both.

3.3.1 HPLC-MS/MS determination of water-soluble vitamins

During the optimization procedure for the separation of the seven WSV studied, the performance of two different reversed phase columns was tested. A short UPLC C_{18} column (50 x 2.1 mm, 1.9 μm particle diameter) and a longer C_{18} column (100 x 2.1 mm, 3 μm particle size) were compared. The longer column provided a better separation among the tested compounds, including those that were weakly retained in the C_{18} stationary phase like ascorbic acid (vitamin C) and thiamine (vitamin B_1), while maintaining short analysis times (7 min). In Figure 3.2, the SRM chromatograms of the 7 WSV studied and hippuric acid are shown. As it can be observed, the separation of all the studied compounds was appropriately achieved. Although other type of columns adequate to perform HILIC separations had been used to separate WSV [5], according to Goldschmidt and Wolf [5], thiamine (B1), pyridoxine (B6) and nicotinamide (B3) were the only vitamins that could benefit from this type of separations. Thus, considering the wider group of WSV studied here, the applicability of the HILIC approach in the present development would be of limited value.

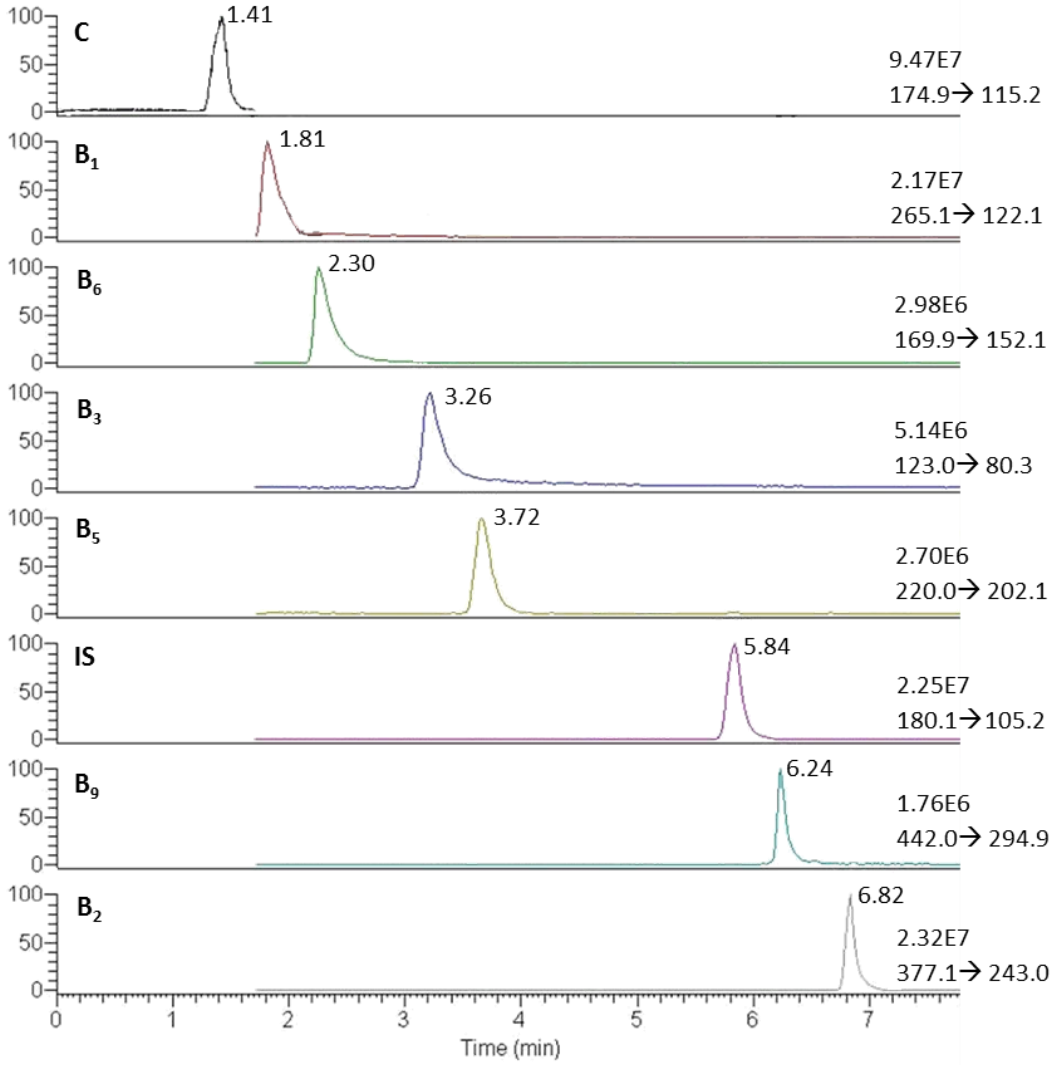

Figure 3.2. HPLC-MS/MS chromatogram of the seven WSV standards and the IS (hippuric acid) under the optimum analysis conditions.

Although the method was developed based on a previously published method [19] using 10 mM ammonium acetate (pH 4.5) and methanol as mobile phases, other alternative mobile phases were also tested. The use of acidified water (using 1% of either acetic or formic acid) did not improve the obtained results using the buffered aqueous mobile phase. Regarding the organic modifier, acetonitrile was also tested. This solvent has been used in several multivitamin determination methods present in the literature [5,11,23], although in this application the separation was not improved. Thus, the use of methanol was maintained. The acidification of the solvent could be useful to suppress the dissociation of vitamins with an acidic group, like ascorbic acid, pyridoxine, pantothenic acid and folic acid (vitamins C, B_6, B_5 and B_9, respectively), improving peak shapes, and also to promote a better ionization of the basic sites of all vitamins [8]. Acetic acid

provided better peak shapes than formic acid. Consequently, different levels of acetic acid were tested, namely 0.1% and 0.3%. It was observed that the higher proportion of acid improved the last eluting peaks, whereas the less retained compounds lost some efficiency. Thus, it was decided to employ a ternary system, allowing the introduction of more acidified methanol as the proportion of the organic modifier increased during the separation (see Table 3.1). Under these conditions, sufficient resolution between peaks and good peak shapes were obtained for all the studied compounds, as it can be observed in Figure 3.2.

Once the separation was optimized, the different MS/MS detection parameters were studied. By using the direct infusion of standard solutions, both positive and negative ESI ionization modes were studied for the production of characteristic precursor and product ions of each compound. Precursor ions were selected as the most abundant mass-to-charge (m/z) values. Subsequently, two product ions for each precursor were chosen. The most intense product ion was used for the quantification whereas the other was employed to confirm the identity of the studied compounds. Table 3.2 shows the precursor and product ions selected for each compound as well as the collision energies and tube lens offset values employed for their detection. As it can be observed, only ascorbic acid (vitamin C) showed more intense ions in the negative ESI ionization mode. The rest of vitamins as well as the internal standard were detected as $[M+H]^+$. Thiamine (vitamin B_1) was monitored as the loss of associated chloride, whereas calcium pantothenate (vitamin B_5) was determined as pantothenic acid. The rest of parameters involved in the ESI detection of the studied vitamins, namely, capillary temperature (250-350 ºC), spray voltage (3000-5000 V), sheath gas pressure (20-60 a.u.) and auxiliary gas pressure (0-40 a.u.), were optimized using a univariate method. The values that provided the best response for all vitamins are described in section 3.2.4.

Once the optimum separation conditions and MS/MS detection parameters were selected, the instrumental intra-day and inter-day precision was evaluated. Intra-day precision was assessed through the consecutive injection (n=5) of a mixture of the seven WSV standards consecutively in the same day, that was repeated for three different days for inter-day precision evaluation (n=15). The obtained results are summarized in Table 3.3. The RSD values, for the same day, ranged between 0.9% and 7.2 % for peak areas and between 0.1 and 2.0% for retention times. The RSD values between days were slightly higher (3.6-9.1% for peak areas and 0.3-1.5% for retention times), although they were always below 10% for peak areas and 2% for retention times (see Table 3.3). The linear range was also tested using a least square fit, which showed a good linearity ($R^2 >$ 0.99) for all WSV, within the range selected. Instrumental LODs (0.18-42.28 ng/ml)

calculated as three times the signal-to-noise (S/N) ratio and LOQs (0.56-128.13 ng/ml) obtained for each WSV as ten times the S/N ratio, are also presented in Table 3.3. These data verified the suitability of the optimized HPLC‑MS/MS method for a rapid and sensitive detection of the 7 WSV in a single run.

3.3.2 HPLC-DAD determination of fat-soluble vitamins

The method chosen to determine the free fat-soluble vitamins, i.e., provitamin A (β-carotene) and vitamin E (α-tocopherol), was based on a previously published method [20,24] with some modifications. A reversed phase column with C_{30} stationary phase was employed to separate the compounds present on the lipophilic extract. This type of column has demonstrated to be capable to separate different geometric isomers of both carotenoids and tocopherols in different matrices [14,25-27]. In fact, compared to C18 stationary phases, the less polar C_{30} phases exhibit superior selectivity for isomer separation of carotenoids, and vitamins A and E [28]. Starting from the conditions already published [20,24], the gradient elution program was modified in order to avoid coelutions between α-tocopherol and some carotenoids present on the real samples. An isocratic period was included at the beginning of the elution, which significantly improved the separation. The particular conditions employed are described in section 3.2.4.

Besides, the DAD detector was sensitive enough for both FSV analyzed, within the concentration range expected in the studied samples (see Table 3.3). Although identification and quantification of the selected compounds was achieved by comparison with their corresponding commercial standards, the DAD detector also permitted the spectral analysis of other compounds present in the extract to identify other tocopherols and carotenoids based on spectra similarity. The intra-day (n=5) and inter-day (n=15, 3 days) values were also assessed. Low RSD (%) values were obtained for both parameters being always below 2.1 and 0.6 % for peak areas and retention times, respectively (see Table 3.3). The linear calibration curves showed a high coefficient of determination (R^2> 0.999).

Table 3.3 Method evaluation parameters: LOD and LOQ values, repeatability, linearity and mean recovery percentages

Vitamin	Detection	LOD (ng/ml)	LOQ (ng/ml)	Repeatability (RSD %)				Linearity		Mean Recovery		
				Intra-day		Inter-day		Range tested	R^2	added	%[a]	RSD
				Area	t_R	Area	t_R	(ng/ml)		(µg/ml)		
Ascorbic Acid (C)	MS/MS	42.28	128.13	5.6	2.0	6.3	2.0	70 - 5280	0.993	3.30	102.8	10.6
Thiamine (B_1)	MS/MS	0.79	2.41	3.1	0.7	3.6	0.7	1.5 - 960	0.993	0.60	87.3	8.5
Riboflavin (B_2)	MS/MS	0.07	0.20	2.5	0.2	4.3	0.2	0.9 - 1500	0.996	0.75	93.9	7.4
Nicotinamide (B_3)	MS/MS	4.35	13.17	2.3	0.5	9.8	0.5	4 – 2800	0.995	1.75	85.4	3.6
Pantothenic Acid (B_5)	MS/MS	7.67	23.25	0.9	0.6	9.1	0.6	20 - 1670	0.998	0.83	88.2	12.6
Pyridoxine (B_6)	MS/MS	0.18	0.56	1.7	0.6	9.1	0.6	0.8 – 530	0.997	0.67	82.8	8.9
Folic Acid (B_9)	MS/MS	0.63	1.90	7.2	0.1	8.4	0.0	0.4 – 530	0.995	0.33	104.8	16.6
Hippuric acid (IS)	MS/MS	4.93	14.9	1.5	0.3	5.7	0.5	3.1 - 5000	0.998	2.50	100.5	4.6
α-tocopherol (E)	UV	170	520	0.7	0.1	2.1	0.5	6250 – 100000	0.999	50	87.5	4.9
β–carotene (pro-A)	UV	70	200	0.4	0.1	0.3	0.2	6250 - 250000	0.999	50	105.3	12.5
β-Apo-8′-carotenal (IS)	UV	17	51	0.5	0.2	1.8	0.4	6250 - 100000	0.999	3.30	88.7	2.5

[a] mean of five extractions; t_R, retention time.

3.3.3 Optimization of the extraction procedure

A sequential and simple method for the extraction of free forms of WSV and FSV from different vegetables is proposed in this work. The method is based on the sequential extraction of the plant material using ultrasounds. The method optimization was carried out following a univariate procedure in order to achieve the maximum extraction of the free forms of the studied vitamins in the shortest total time. Basically, the freeze-dried material was mixed with a volume of 10 mM ammonium acetate (pH 4.5)/methanol (50:50, v/v) containing 0.1 % BHT in order to obtain the water-soluble vitamins. The use of methanol in the mixture allowed the better dissolution and extraction of the less polar studied WSV [8,21]. On the other hand, BHT was included to prevent the oxidation of the some vitamins like vitamin C or β-carotene and α-tocopherol. Despite the fact that BHT is not soluble in water and is normally used to protect fat soluble compounds, the inclusion in this stage protected the sample from oxidation since the beginning of extraction. Although this was not the best stabilizer for vitamin C, its presence has been described to partly inhibit its oxidation [8]. Other chemicals might potentially have a higher protective effect in this process, like metaphosphoric acid or EDTA, but their presence is also referred to cause ion suppression in MS detection [8,29]. Thus, the combined use of BHT and methanol, provided a compromise for all the studied vitamins. A 15 min ultrasonic extraction was performed to promote the dissolution of the vitamins from the vegetal matrix into the solvent. Due to the labile nature of most vitamins, the temperature of the ultrasound bath was always controlled, and all procedures were performed without direct exposure to light. After centrifugation of the extracts, the supernatant was evaporated under a N_2 stream. The solid residue was then re-extracted for 15 min using ethyl acetate as extracting solvent to recover FSVs.

To determine the optimum volume of solvents and the number of extraction cycles suitable for all vitamins, optimization was carried out with spiked samples. Concerning the extraction of WSV, 16 ml was fixed as solvent volume and the possibility of using an extraction step or two extraction steps (8 ml each) was studied (triplicate assays). As it can be observed in Figure 3.3A, using two consecutive cycles, the amount of extracted vitamins was slightly higher, although the differences were not statistically significant ($p > 0.05$). Thus, a single extraction step was selected in order to make the whole procedure faster. On the other hand, different volumes of ethyl acetate (8, 12 and 15 ml) were tested for the FSV extraction. Results obtained showed an improvement in the final results when 12 ml of solvent were used compared to 8 and 15 ml. Similarly to WSV, the possibility of dividing the extraction volume in different cycles was studied. It was observed that the use

of 2 extraction cycles (6 + 6 ml) significantly improved the results obtained compared to just one extraction cycle (see Figure 3.3B). The use of a third extraction cycle was also explored but discarded considering that did not produce significant gains while increasing the total extraction time. The extracts obtained were concentrated under a N_2 stream. This step was especially necessary for the α-tocopherol detection, due to its low signal in the DAD detector.

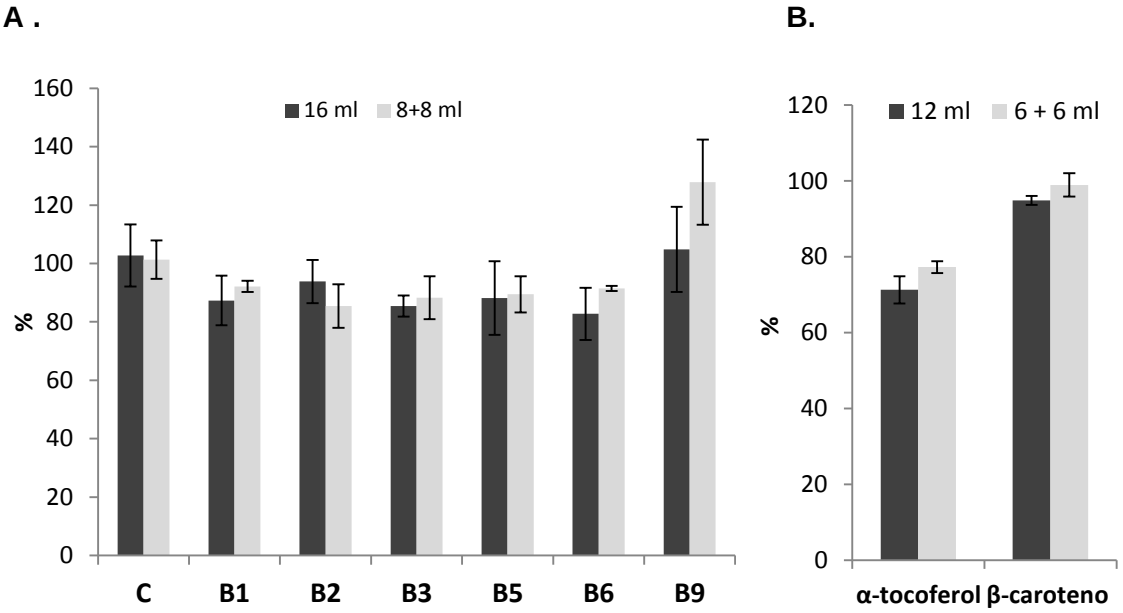

Figure 3.3 A) Recoveries of water-soluble vitamins (%) obtained after 1 *vs* 2 extraction cycles (16 ml *vs* 8±8 ml). B) Recoveries (%) of fat-soluble vitamins obtained after the one (12 ml) and two (6 + 6 ml) extraction cycles.

Concerning the determination of FSV, a hot saponification procedure is often used to remove chlorophylls and lipids while allowing the release of the free vitamin forms that are found as esters. However, in our approach, this procedure was not suitable due to the degradation of carotenoids by thermal isomerization and the generation of some artifacts that can interfere in the vitamin's determination. Moreover, this step is not recommended for green leafy vegetables, tomatoes and carrots, due to their low contents of FSV esters [14,30]. Instead, the chlorophylls found in the studied vegetables were, in our optimized method, properly separated from the target compounds during the chromatographic analysis.

3.3.4 Extraction recovery study

To assess the suitability of the complete method, a recovery study was performed. Namely, 2 sets of extractions were carried out simultaneously following the optimized

procedure described in section 3.2.3. In one of them, a vegetable sample (spearmint) spiked with a known amount of the target compounds was extracted whereas in the other, the same sample was extracted and spiked with the same concentration of vitamins after extraction. Triplicates of the extraction protocols were performed and the extracts were analyzed using the two methods by duplicate. Comparing the values obtained, it was possible to deduce that no significant matrix effect was observed during the extraction (Figure 3.4). The recoveries ranged between 83 % and 105% (see Table 3.3) which was considered as appropriate for a method that simultaneously determines compounds with different structures and chemical properties.

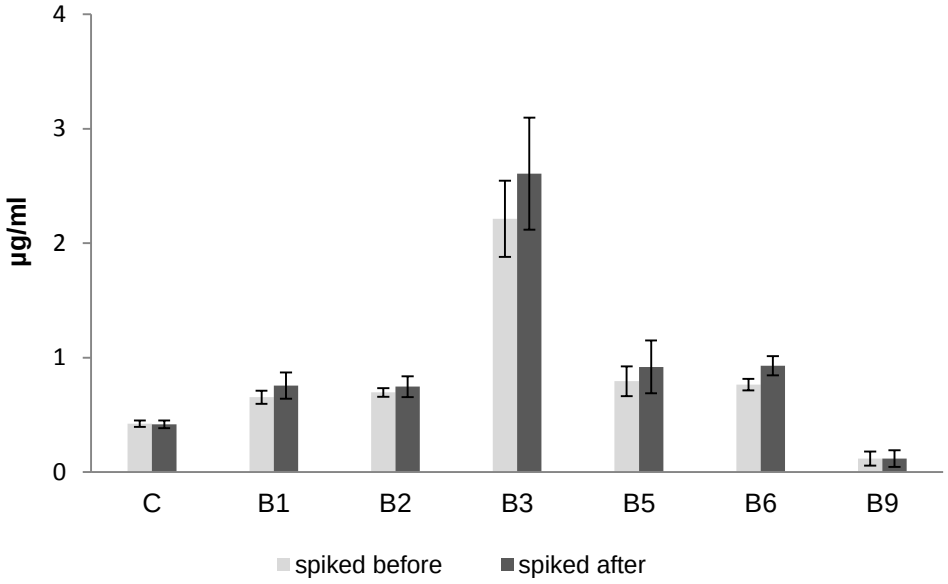

Figure 3.4 Mean content of water-soluble vitamins of sample spiked with the same amount of WSV standards before and after extraction.

3.3.5 Green leafy vegetables analysis

Once the complete method of extraction and analysis of WSV and FSV was optimized, the procedure was applied to the study of different fresh-cut green leafy vegetables. The aim was to determine how the vitamin contents evolved during storage under conditions similar to those found in the market. To do that, 12 different products (namely, green lettuce, ruby red lettuce, wild rocket, swiss chard, watercress, spinach, lamb's lettuce, spearmint, pea leaves, mizuna, red mustard and garden cress), for which there were not previous published results, were analyzed upon arrival and after a 10-days storage at 3 ºC. The results obtained from these determinations are summarized in Table 3.4, expressed as μg of vitamin per 100 g fresh weight (fw).

Table 3.4 WSV and FSV content (free forms) found in the fresh-cut green leafy vegetables at the indicated storage times. Values shown as mean ± sd relative to fresh weight (fw). Asterisks indicate values not statistically different ($p > 0.05$) between day 1 and day 10.

SAMPLES	Ascorbic Acid (C) mg/100g fw±sd	Thiamine (B_1) µg/100g fw±sd	Riboflavin (B_2) µg/100g fw±sd	Nicotinamide (B_3) µg/100g fw±sd	Pantothenic Acid (B_5) µg/100g fw±sd	Pyridoxine (B_6) µg/100g fw±sd	Folic Acid (B_9) µg/100g fw±sd	α-tocopherol (E) mg/100g fw±sd	β-carotene (provit A) mg/100g fw±sd
Green Lettuce									
day 1	0.1 ± 0.0*	79 ± 27*	28 ± 4*	130 ± 20	147 ± 24	2 ± 0.2	n.d.	n.d.	5.5 ± 0.3
day 10	0.1 ± 0.0*	80 ± 3*	30 ± 5*	80 ± 6	215 ± 22	3 ± 0.3	n.d.	n.d.	2.6 ± 0.2
Ruby Red Lettuce									
day 1	0.1 ± 0.0*	68 ± 15*	28 ± 4	195 ± 13	77 ± 19	15 ± 2	n.d.	1.0 ± 0.1	3.4 ± 0.0
day 10	0.1 ± 0.0*	55 ± 6*	18 ± 3	55 ± 3	127 ± 16	6 ± 0	n.d.	0.6 ± 0.0	2.0 ± 0.0
Wild Rocket									
day 1	85.1 ± 5.7	11 ± 2	43 ± 13*	143 ± 19	309 ± 75	10 ± 1	n.d.	1.6 ± 0.1*	9.3 ± 0.9
day 10	3.6 ± 0.7	24 ± 0.8	54 ± 4*	85 ± 6	790 ± 15	28 ± 2	n.d.	1.4 ± 0.0*	6.8 ± 0.6
Swiss Chard									
day 1	n.d.	14 ± 2	120 ± 6	201 ± 12	247 ± 7	22 ± 1	n.d.	1.2 ± 0.1*	7.2 ± 0.2
day 10	n.d.	11 ± 2	111 ± 4	139 ± 19	490 ± 42	46 ± 3	n.d.	1.0 ± 0.1*	6.3 ± 0.2
Watercress									
day 1	59.6 ± 2.3	70 ± 21*	143 ± 7	111 ± 10	263 ± 3	13 ± 0.1*	n.d.	2.5 ± 0.2	8.3 ± 0.0
day 10	48.2 ± 0.8	99 ± 11*	163 ± 9	77 ± 5	560 ± 63	16 ± 2*	n.d.	0.3 ± 0.1	9.3 ± 0.1
Spinach									
day 1	14.4 ± 2.8	194 ± 14	257 ± 21	167 ± 12*	345 ± 20	8 ± 0.2	1 ± 0.1*	2.9 ± 0.1	11.2 ± 1.2*
day 10	1.7 ± 0.0	244 ± 24	223 ± 17	179 ± 15*	526 ± 59	13 ± 0.6	1 ± 0.1*	3.6 ± 0.3	13.4 ± 1.5*
Lamb's lettuce									
day 1	59.1 ± 10.0	131 ± 6*	169 ± 32	241 ± 46*	585 ± 38	20 ± 5	11 ± 0.6	3.1 ± 0.2*	9.2 ± 0.1*
day 10	13.7 ± 8.3	130 ± 13*	111 ± 8	194 ± 5*	698 ± 45	9 ± 1	6 ± 1	3.2 ± 0.4*	9.4 ± 0.1*
Spearmint									
day 1	0.5 ± 0.0	121 ± 10	169 ± 25*	282 ± 7	557 ± 31	8 ± 0.9	n.d.	1.4 ± 0.0*	10.6 ± 1.3
day 10	0.3 ± 0.0	216 ± 13	144 ± 14*	239 ± 13	1622 ± 118	5 ± 0.1	n.d.	1.5 ± 0.2*	8.8 ± 0.0
Pea leaves									
day 1	154.0 ±2.8*	178 ± 15*	112 ± 14*	131 ± 2	637 ± 30	20 ± 1	n.d.	2.7 ± 0.1	14.4 ± 0.1*
day 10	174.0 ± 19.3*	189 ± 6*	128 ± 11*	104 ± 5	1186 ± 33	48 ± 1	n.d.	3.7 ± 0.1	13.6 ± 0.6*
Mizuna									
day 1	33.0 ± 1.6	6 ± 0.5	53 ± 5*	58 ± 8	125 ± 11	8 ± 0.5	n.d.	1.1 ± 0.0	7.8 ± 0.2*
day 10	14.7 ± 1.2	25 ± 4	56 ± 5*	35 ± 3	693 ± 47	21 ± 3	n.d.	2.7 ± 0.0	9.4 ± 0.8*
Red Mustard									
day 1	43.9 ± 1.1	12 ± 2	64 ± 4	108 ± 13	113 ± 7	5 ± 1*	n.d.	2.1 ± 0.2*	8.2 ± 0.1
day 10	0.1 ± 0.0	18 ± 2	28 ± 1	67 ± 6	278± 23	4 ± 0.7*	n.d.	1.9 ± 0.0*	6.3 ± 0.0
Garden Cress									
day 1	10.1 ± 1.2	36 ± 3	125 ± 10*	191 ± 21	587 ± 18	4 ± 0.2	n.d.	1.9 ± 0.2	12.7 ± 0.2
day 10	0.2 ± 0.0	102 ± 5	122 ± 3*	159 ± 15	1222 ± 100	17 ± 1	n.d.	2.5 ± 0.1	10.8 ± 0.1

As it can be observed, the vitamins found in highest amounts in general were provitamin A, ascorbic acid and vitamin E. The amounts of vitamin C found in the samples varied in great extent, from more than 150 mg/100 g fw in pea leaves to undetectable amounts in swiss chard. Together with pea leaves, garden cress and wild rocket were the richest samples on this component. For most of the samples, the contents of vitamin C significantly decreased after the 10-days storage. Very strong decreases were observed for red mustard and garden cress. Other samples, such as wild rocket or watercress did not experiment these great losses. Interestingly, a slight but no statistically significant increase ($p > 0.05$) was observed for pea leaves, whereas a significant increment was found for lamb's lettuce. It has been shown that over a storage period similar to that employed in this work, ascorbic acid present in fresh-cut vegetables can be oxidized to dehydroascorbic acid as a result of the function of this compound in the plant as protector against oxygen species [31], even regenerating tocopherols [32]. Vitamin C could be also responsible for the decrease in enzymatic browning observed in some fresh-cut vegetables during storage [33].

B-group vitamins were found in less amount in all the studied vegetables, as it could be expected considering that most of B vitamins are mainly contained in higher extent in animal-derived foods and cereals. Among them, pantothenic acid (vitamin B_5) was generally the richest in all the studied samples. In fact, in all cases, the amount of free vitamin B_5 detected in the samples after the 10-days storage period was always significantly higher than the contents found before. These increments were in the range of a 2-fold increase, although for some vegetables, the increase was even higher (e.g., mizuna).There are several possible explanations to this observation; on one hand, a catabolic release of pantothenic acid from coenzyme A (CoA) and acyl carrier protein (ACP) mediated by a chain of enzymatic steps including hydrolases (for ACP) or phosphatases and pyrophosphatases (for CoA) [34] thus freeing the vitamin B_5 from CoA; and on the other hand an increase on the whole vitamin B_5 content due to a higher vitamin B_5-bacterial producing microbiological load of the green leafy ready-to-eat vegetables after storage. A recent study showed an increase in bacterial load of fresh-cut salads commercialized in Portugal [35] that might be susceptible of synthesizing higher amounts of this vitamin. In fact, processes associated to fresh-cut products such as washing, cutting, shredding and slicing have been identified as potential sources of microbial contamination [36]. An increase as a result of the post-harvest metabolism should not be also discarded [37].

Folic acid (vitamin B_9) was only found in spinach and lamb's lettuce, at levels lower than 11.5 μg/100 g; the contents on folic acid in spinach were by far lower than those

reported in other published works [38], although it should be kept in mind that only the form as folic acid was determined in the present work. On the other hand, pyridoxine (vitamin B_6) was found in all the studied samples, although maintaining very low levels, even as low as 1.5 μg/100 g. In general, the amount of vitamin B_6 after storage was lower than before, with some exceptions. Even in those latter cases, the differences were small.

For the rest of B-group vitamins, the levels varied among samples. Generally, nicotinamide (vitamin B_3) was more abundant than riboflavin and thiamin (vitamins B_2 and B_1, respectively) in almost all samples. For some samples, the contents on these vitamins increased after the 10-days storage time, mainly for vitamin B_1 (see wild rocket, spinach, spearmint, mizuna and garden cress in Table 3.4). Nevertheless, these increments were not too high considering the amounts of these free vitamins found in the studied samples. Similar behaviors have been observed for other vegetables at the end of prolonged storage periods [39]. The increments in some of them, such as vitamin B_2, might be related to microbial growth in the samples [39]. Besides, other authors have observed post-harvest synthesis of these vitamins, which could also explain the increments found [37]. Figure 3.5 shows the corresponding chromatograms obtained during the quantification of the studied vitamins in garden cress. As it can be observed in Figure 3.5B, some other compounds could be tentatively assigned to carotenoid or tocopherol family, thanks to the use of a DAD together with the high resolving power of the C_{30} stationary phase employed in this determination.

The levels of fat-soluble vitamins found in the samples were by far higher than those of the B-group vitamins. Provitamin A (β-carotene) was the most abundant FSV in all samples reaching maximum values for pea leaves, garden cress and spinach. The poorest source of provitamin A was ruby red lettuce, with values lower than 3.5 mg/100 g fw. For 4 out of the 12 samples studied, the levels of provitamin A did not varied significantly after the 10-days storage period, namely, mizuna, pea leaves, lamb's lettuce and spinach. For other samples, small to moderate decreases were observed, being green lettuce the fresh-cut vegetable that presented a higher percentage of loss of its initial content on this provitamin (ca. 50 %).

The amount of vitamin E (α-tocopherol) in the studied vegetables after storage ranged from 321 μg/100 g fw for watercress to 3663 μg/100 g fw for pea leaves. A statistically significant increase ($p < 0.05$) on the concentration of vitamin E after storage was detected in spinach, pea leaves, mizuna and garden cress. These samples were also among the richest on vitamin C, which, as it has been already mentioned, is able to regenerate vitamin E [32].

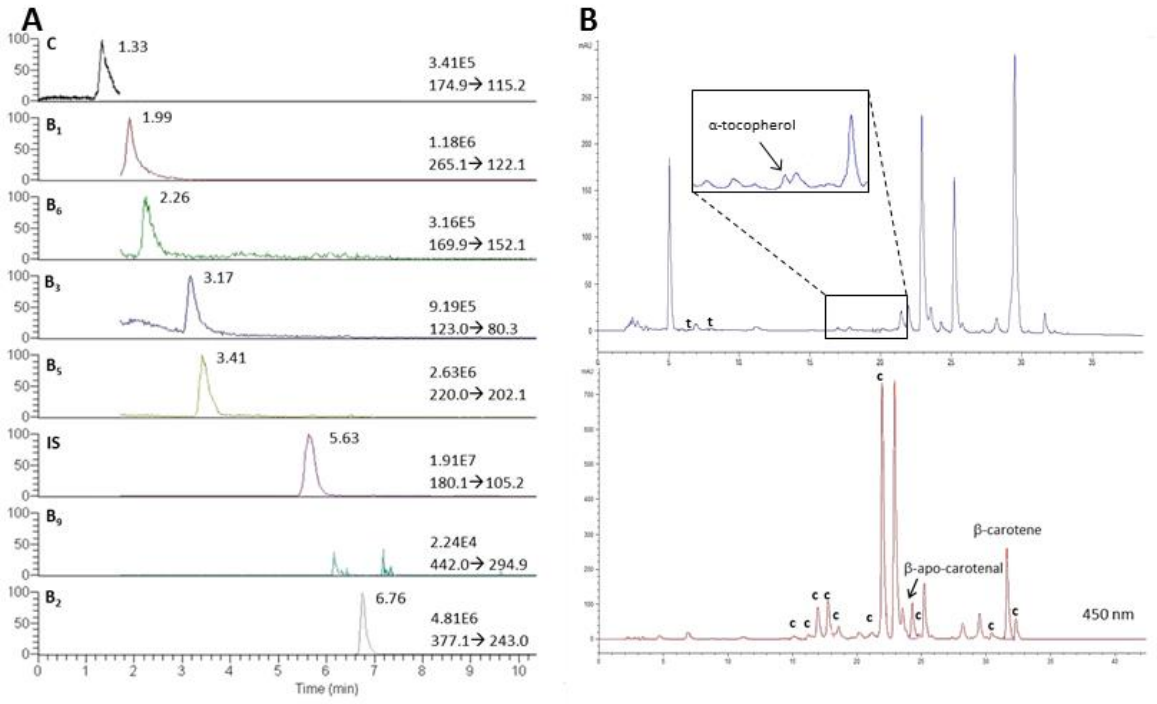

Figure 3.5 Chromatograms of WSV (A) and FSV (B) from an extracted sample (garden cress). Peak id: t: tocopherols; c- carotenoids.

3.4 Conclusions

In this study, a methodology to extract and quantify several free water soluble vitamins (vitamins C, B_1, B_2, B_3, B_5, B_6 and B_9) and fat soluble vitamins (vitamin E and provitamin A) by LC-MS/MS and LC-DAD, respectively, has been optimized. The method has been used to quantify the vitamin's level in 12 different fresh-cut vegetables before and after a 10-days storage period under refrigeration (3ºC). The optimized sequential extraction-analysis procedure has revealed as an appropriate methodology for the simultaneous determination of a whole range of free water and fat-soluble vitamins, with different chemical structures, in real complex samples. The procedure allows a complete analysis of all the vitamins in less than 100 min of total analysis time, including extraction and determination, with recoveries ranging from 83 to 105%, suitable LOD and LOQ and appropriate intra-day and inter-day precisions.

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CHAPTER 4.

Multi-elemental analysis of ready-to-eat “baby leaf” vegetables using microwave digestion and high-resolution continuum source atomic absorption spectrometry

This chapter presents the development and validation of a HR-CS-AAS method for mineral analysis in baby leaf vegetables. The mineral profile of the baby leaf was compared with mature vegetables. Further comparisons of the overall mineral composition were made through a Linear Discriminant Analysis.

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Multi-elemental analysis of ready-to-eat "baby leaf" vegetables using microwave digestion and high-resolution continuum source atomic absorption spectrometry

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Abstract

The mineral content (phosphorous (P), potassium (K), sodium (Na), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu)) of eight ready-to-eat baby leaf vegetables was determined. The samples were subjected to microwave-assisted digestion and the minerals were quantified by High-Resolution Continuum Source Atomic Absorption Spectrometry (HR-CS-AAS) with flame and electrothermal atomization. The methods were optimized and validated producing low LOQs, good repeatability and linearity, and recoveries, ranging from 91% to 110% for the minerals analysed. Phosphorous was determined by a standard colorimetric method. The accuracy of the method was checked by analysing a certified reference material; results were in agreement with the quantified value. The samples had a high content of potassium and calcium, but the principal mineral was iron. The mineral content was stable during storage and baby leaf vegetables could represent a good source of minerals in a balanced diet. A linear discriminant analysis was performed to compare the mineral profile obtained and showed, as expected, that the mineral content was similar between samples from the same family. The Linear Discriminant Analysis was able to discriminate different samples based on their mineral profile.

Keywords: baby leaf vegetables, minerals, HR-CS-AAS, microwave assisted digestion, LDA.

4.1 Introduction

Vegetables have almost all essential nutrients for human metabolism, if consumed as recommended, and are linked with the promotion of good health (Miller-Cebert, Sistani & Cebert, 2009). Baby leaf salads are a relatively new ready-to-eat product, which is gaining popularity among the consumers (Clarkson, O'Byrne, Rothwell & Taylor, 2003). It is prepared with young leaves, harvested at a very early stage of maturation while still metabolically active. These leaves offer a softer texture and add a variety of colours and shapes to the meal (Martínez-Sánchez et al., 2012). They have an appealing appearance due to their 3-D structure, and experience lower levels of oxidative damage because of their small stem diameter. These products have greater stability during shelf life, despite being packed without modified atmosphere or any other compounds to delay deterioration. They rely, almost exclusively, on strict control of storage temperature to preserve quality (Martínez-Sánchez et al., 2012; Wagstaff et al., 2007). Lowering the temperature of the product will slow the leaf metabolism, decreasing their respiratory rate (Rico, Martín-Diana, Barat & Barry-Ryan, 2007). Minimal processing (washing, cutting and packaging) increases vegetables metabolic rate, leading to faster deterioration, which could result in rapid loss of components influencing flavour and nutritional value (Conte et al., 2008; Martínez-Sánchez et al., 2012). Maturity can also affect composition and the stability of fresh-cut vegetables (Martínez-Sánchez et al., 2012).

Accurate nutritional information is needed by public agencies and agricultural/ food industries to promote products and healthy eating (Borah, Baruah, Das & Borah, 2009; Miller-Cebert et al., 2009). Although green leafy vegetables are less rich in protein and carbohydrates, they are a good source of most minerals and vitamins (Altundag & Tuzen, 2011; Grusak & DellaPenna, 1999). Minerals are among the micronutrients that must be obtained through diet (Reddy & Bhatt, 2001). The basic functions of minerals in biological systems include participation in proteins, lipids and carbohydrates metabolism, as well as cellular and skeletal structure and a role in osmotic pressure and acid/base regulation (Chekri et al., 2012). Plants accumulate minerals accordingly to their requirements; however, the mineral content can be affected by genetic factors, soil and weather conditions, the use of fertilizers, and the plant’s maturity at harvest (Grusak & DellaPenna, 1999; Sanchez-Castillo et al., 1998). Therefore, mineral quantification of vegetables must be done to assure the nutritional values stated on package label.

Mineral analyses in vegetables are common, and there are numerous techniques described in the literature. These include Atomic Absorption Spectrometry (AAS) (Chekri et al., 2012) with flame (FAAS) or electrothermal (EAAS) atomization and the Inductively

Coupled Plasma Spectrometry (ICP) (Cooper et al., 2011) with atomic emission spectrometry (ICP-AES) (Lisiewska, Gębczyński, Bernaś & Kmiecik, 2009), optical emission spectrometry (ICP-OES) (Altundag & Tuzen, 2011; Miller-Cebert et al., 2009), or mass spectrometry (ICP-MS) (Noël, Leblanc & Guérin, 2003). Although, AAS is not the newest technique, its use has expanded with the development of High-Resolution Continuum Source Atomic Absorption Spectrometry (HR-CS-AAS) (S. R. Oliveira, Neto, Nóbrega & Jones, 2010; Welz, 2005). The equipment has a continuous radiation source between 190 to 800 nm and a double mono-chromator with double-Echelle grating, which allows rapid change of wavelength during a sequential multi-element analysis. The linear CCD array detects not only the analytical line, but also, its spectral environment at high resolution, producing an atomic spectrum with 200 pixels. The central pixels correspond to the analyte line, including the spectral environment of about ± 0.2 nm around that chosen analyte line, i.e. the noise. This feature allows monitoring the baseline and avoids signal interferences by correcting the baseline automatically, creating a very stable system with low noise levels and significantly lower detection limits (S. R. Oliveira et al., 2010; Welz, 2005). Although there are a few studies using HR-CS-AAS for food analysis (M. Oliveira et al., 2012; S. R. Oliveira et al., 2010; Welz, 2005), determination of a wide group of macro- and micro-minerals in green baby leaf vegetables has not been done previously. Therefore, the aim of this study was to (i) optimize and validate a HC-CS-AAS method for determining macro- (K, Na, Ca and Mg) and micro- (Fe, Mn, Zn and Cu) mineral composition of ready-to-eat “baby leaf”, which included microwave mineralization of the samples; (ii) study the mineral content (phospurous, potassium, sodium, calcium, magnesium, iron, manganese, zinc and copper) of different green “baby leaf” vegetables; and (iii) the stability of the mineral content during a 10 days storage period.

4.2 Materials and methods

4.2.1 Reagents and analytical solutions

The ultrapure water (18.2 MΩ.cm resistivity) used was from a Simplicity 185 water purification system (Millipore, Molsheim, France). Standard solutions of potassium, calcium, magnesium, iron, manganese, zinc and copper were prepared from 1000 mg/L stock solutions (Panreac Quimica SA, Barcelona, Spain). The phosphorous standard solution was made using potassium dihydrogen phosphate from Riedel-de Haën (Seelze, Germany) and the sodium from the 1000 mg/L stock sodium chloride from Merck (Darmstadt, Germany). Standards and samples solution were acidified with 1% (v/v) of

65% nitric acid (Sigma-Aldrich, Steinheim, Germany), except for phosphorous analysis. To avoid ionization and chemical interferences in potassium, sodium, calcium, magnesium, iron, manganese and zinc HR-CS-FAAS analysis, caesium chloride (1% w/v; Sigma–Aldrich, Steinheim, Germany) was used as ionization buffer. To avoid interference from elements that could form stable oxy-salts and enhance sensitivity, 1% (w/v) of $LaNO_3$ (Panreac Quimica SA, Barcelona, Spain) was added to the standards and samples solution for calcium determinations. Copper was analysed by HR-CS-EAAS using a matrix modifier prepared from the relevant salts ($Pd(NO_3)_2$ (0.1%), $Mg(NO_3)_2$ (0.05%) in water (Merck, Darmstadt, Germany and Panreac Quimica SA, Barcelona, Spain, respectively)). Phosphorous was quantified using a spectrometer method according to 4500-P standard method (Greenberg, Clesceri & Eaton, 1992); the colour development reagent was prepared with ammonium molybdate tetrahydrated (99.0%) and ammonium metavanadate (99.0%) (Merck, Darmstadt, Germany). All glassware and plastic material were soaked in 10% nitric acid for 24 h, rinsed with ultra-pure water and dried before use.

4.2.2 Samples

Minimally processed baby leaf vegetables (washed and packaged) were supplied by a producer (Odemira, Portugal). The samples were received at the laboratory one day after processing. The samples were baby leaves of green lettuce (Lactuca sativa var. crispa), swiss chard (Beta vulgaris), watercress (Nasturtium officinale), lamb's lettuce (Valerianella locusta), wild rocket (Diplotaxis muralis) from conventional and organic production, spinach (Spinacia oleracea) and parsley (Petroselinum crispum). About 1 kg of baby leaf samples were freeze-dried (Telstar Cryodos-80, Terrassa, Barcelona), on arrival and after 10 days of refrigerated storage (3 ± 1 ºC, monitored with a EL-USB 2, Lascar Electronics, Salisbury, UK). The freeze-dried leaves were reduced to a fine powder in a knife mill (GM 200, RETSCH, Haan, Germany) and stored protected from light, oxygen and high temperatures. This procedure generated a composite sample to exclude individual differences and ensure that the test sample was representative.

4.2.3 Microwave digestion

The sample mineralization was carried out by microwave assisted digestion MARS-X (CEM, Mathews, NC, USA). The procedure was adjusted to achieve complete mineralization of the sample within the shortest time possible. The microwave program (Table 4.1) was adjusted using 0.1 g of the freeze dried wild rocket baby leaf digested with 9 mL of nitric acid. A four stage program with a maximum temperature of 180 ºC was

chosen. Portions of the samples (0.1, 0.15 and 0.2 g of freeze-dried baby leaf) and mixtures of nitric acid/ultrapure water (9:0; 6:3; 7:2; 5:4) and nitric acid/hydrochloric acid (7: 2) were tested. The best conditions were 0.15 g of freeze dried sample, nitric acid/ultrapure water (6:3) and 15 minutes prior to microwave digestion. Samples were quantitatively transferred to a graduated plastic tube and diluted to a final volume of 15 ml with ultrapure water. Four replicates of each sample from the two sampling days (day 1 and day 10) were digested. Blank digestion was carried out without addition of any sample. A certified reference material BCR ®- 679 (freeze dried white cabbage), obtained from Institute of Reference Materials and Measurements, was digested and analysed with the same protocol.

Table 4.1 Microwave digestion program

Stages	**1**	**2**	**3**	**4**
Power (W)	600	600	600	600
Time (min.)	3	5	8	2
Temperature (control)	50ºC	90ºC	170ºC	180ºC
Hold (min.)	5	10	20	10

4.2.4 Mineral analysis

Potassium, sodium, calcium, magnesium, iron, manganese and zinc were analysed using a HR-CS-FAAS Analytik Jena ContrAA 700, equipped with a xenon short-arc lamp of 300 W (XBO 301, GLE, Berlin, Germany), operating in a hot-spot mode. An air/acetylene oxidising flame (Linde, Portugal) was used and the equipment was coupled to an AS52S autosampler (Analytik Jena, Germany). Copper was analysed in the same equipment with a graphite furnace module equipped with an MPE60 autosampler (Analytik Jena, Germany) and argon as the inert gas (Linde, Portugal). The more sensitive line (primary line) of each mineral was chosen and external standard calibration achieved with five standard solutions. The best instrumental analytical parameters were tested and tuned with standards solutions for each mineral (see Table 4.2). The most adequate pyrolysis and atomization temperatures for copper analysis were 1050 ºC and 1950 ºC, respectively, with 300 ºC/s for pyrolysis ramp and 1500 ºC/s for atomization ramp. Analytical blanks and standards were evaluated daily and frequently along with samples to check instrument performance. All measurements were performed in triplicate. Phosphorous content was determined in the digested solution, according to the 4500-P standard method (Greenberg et al., 1992) in a UV/VIS spectrophotometer at 420 nm (Evolution 300, Thermo Scientific, USA).

4.2.5 Method validation

Certified reference materials for iron, manganese, zinc and copper and post-digested spiked samples were used to assess the accuracy of the method. The recovery studies for each mineral were performed with six baby leaf samples, each one spiked after microwave digestion, with two different amounts of the target minerals (see table 4.3). Instrumental intra- and inter-day precision was assessed by analysing the signal variation of 15 consecutive readings (intra-day) of each mineral at low and at a medium concentration off the linear range (see table 4.3), and on three different days (inter-day). The limits of detection (LOD) and quantification (LOQ) were 3 and 10 times the standard deviations of the blank reagent divided by the slope of the calibration curve, respectively, and expressed in µg/g.

4.2.6 Statistical analysis

Mineral contents were calculated on a fresh weight (fw) basis. Data were expressed as mean ± standard deviation and the differences between mineral contents of the samples and between the two days of sampling (day 1 and day 10) were tested by the one-way ANOVA followed by post-hoc Tukey HSD test. Statistical significance was defined as $p < 0.01$. Also a multivariate analysis of the data was performed in order to have a global perspective (i.e. all minerals and all samples analysed simultaneously). A Linear Driscriminant Analysis (LDA) was performed with forward stepwise analysis, considering a p value of 0.01, to determine which minerals were useful to discriminate between the naturally occurring groups. All variables were reviewed and evaluated at each step, choosing each time, the variable with most significant contribution to the discriminant function model generated. To learn more about the nature of the discrimination, a canonical analysis was performed. This generated coefficients of canonical variates, derived from the maximization of the correlation between canonical variates and the variables in the discriminant model. The statistical analyses were carried out using Statistica 8.0 software (Statsoft Inc., Tulsa, USA).

Table 4.2 Optimized instrumental analytical parameters for the determination of mineral elements by high-resolution continuum source atomic absorption spectrometry

Element	Atomization	Wavelength (nm)	Read time (s)	Pixels	Matrix modifier	FAAS optimized analytical parameters	
						acetylene (L/h)	height burner (mm)
K	Flame	766.4908	3	3	0.1% CsCl	45	6
Na	Flame	588.9953	3	3	0.1% CsCl	40	5
Ca	Flame	422.6720	3	3	0.1% CsCl + 1% $La(NO_3)_3$	50	5
Mg	Flame	285.2125	3	3	0.1% CsCl + 1% $La(NO_3)_3$	50	5
Fe	Flame	248.3270	0.5	3	0.1% CsCl	55	7
Mn	Flame	279.4817	0.5	3	0.1% CsCl	55	7
Zn	Flame	213.8570	3	3	0.1% CsCl	55	6
Cu	Graphite furnace	324.7540	5	3	$Pd(NO_3)_2$ (0.1%) / $Mg(NO_3)_2$ (0.05%)	-	-

Table 4.3 Method evaluation parameters: LOD and LOQ values, linearity, repeatability and mean recovery of the 9 mineral elements

Element	Regression equation[a]	Linearity		LOD	LOQ	Repeatability (RSD %)				Recovery			
		range tested (mg/L)	R^2	(µg/g)[b]	(µg/g)[b]	intraday		interday		Spike 1		Spike 2	
						Level 1*	Level 2**	Level 1*	Level 2**	added (mg/g)[b]	%[c]	added (mg/g)[b]	%[c]
K	y=0.056x + 0.0052	0.5-10	0.999	0.438	1.316	2.01	1.48	1.01	2.34	33.33	101.2±7.7	133.33	108.5±2.5
Na	y=0.677x + 0.0053	0.05-0.75	0.995	0.012	0.035	0.82	0.72	3.09	2.08	13.33	105±11	33.33	106.5±7.6
Ca	y=0.037x - 0.0022	0.25-5	0.999	0.240	0.720	1.87	0.95	5.14	4.03	25.00	109.9±3.5	75.00	105.7±3.2
Mg	y=1.042x + 0.0014	0.02-0.45	0.999	0.008	0.021	1.25	0.99	8.95	3.88	2.62	105±13	7.87	101.4±6.4
P	y=0.074x - 0.0018	0.4-3	0.999	0.436	1.322	0.24	0.28	6.17	2.52	40.00	93.3±2.1	100.0	91.8±4.4
Fe	y=0.065x + 0.0010	0.2-0.25	0.999	0.306	0.900	2.53	1.19	4.21	4.41	0.08	102 ±12	0.33	97.5±5.4
Mn	y=0.188x + 0.0013	0.06-1.5	0.998	0.060	0.180	1.81	0.86	4.01	3.90	0.03	96.2±5.9	0.10	95.7±7.1
Zn	y=0.337x - 0.0361	0.1-0.6	0.995	0.237	0.713	4.15	1.51	9.08	6.56	0.03	98.5±9.1	0.07	101.1±9.0
Cu	y=0.014x + 0.0122	0.0025-0.025	0.997	0.004	0.012	3.11	2.46	11.15	7.67	0.003	97.7±9.1	0.01	103.7±3.3

* Level **1** (mg/L): K 0.8; Na 0.15; Ca 0.5; Mg 0.04; Fe 0.4; Mn 0.12; Zn 0.1; Cu 0.0075; P 0.8.

Level **2 (mg/L): K 6; Na 0.75; Ca 3.5; Mg 0.45; Fe 2.5; Mn 1.2; Zn 0.6; Cu 0.015; P 2.4.

[a] y: absorvance; x: concentration (mg/L for K, Na, Ca, Mg, Fe. Mn, Zn and P; µg/L for Cu)

[b] values represent quantity of mineral /g of sample (dry weight).

[c] mean values ± RSD obtained for the recovery tests of six green leafy vegetables samples (watercress, lamb's lettuce, spinach, parsley, lettuce and wild rocket)

4.3 Results and discussion

4.3.1 Microwave digestion optimization

Microwave digestion is a simple, fast, accurate, precise and cost efficient method (Altundag & Tuzen, 2011). Mineralization is a critical step in most analytical methods for determination of chemical elements in food samples due to possible analyte loss during the process (Aguilar, Cantarelli, Marchevsky, Escudero & Camiña, 2011). Different matrices demand optimization of the mass portion, acid mixture and microwave program to achieve complete digestion. The four stages microwave program (Table 4.1) allowed a gradual increase of the digestion temperature without exceeding the pressure limits of the Teflon vessels used. A total time of approximate 1 hour was chosen to guarantee a complete digestion of all samples, although this time was more than necessary for some samples, particularly those more easily digested. Nitric acid is the most common approach for organic matrix digestion (Miller-Cebert et al., 2009; Noël et al., 2003). However, diluted acid must be used to reduce acidity and protect certain instrument parts (Castro et al., 2009). According to Gonzalez et al. (2009), the use of diluted acids reduces the volume of the reagents needed, permits higher temperatures, prevents the formation of insoluble salts, and improves the solubility of some minerals. The diluted acids produced transparent solutions and a pressure decrease in the control vessel. Comparing the three mixtures of HNO_3: H_2O tested, the 6:3 HNO_3:H_2O registered the best scores (more constant temperature profile during the program, lower pressure values and complete digested solutions). A mass portion of 0.15 g of freeze dried vegetable produced the best results (complete digestion) with the microwave program and acid mixture selected. The digestion procedure was effective for all freeze dried samples analysed and for the certified reference material.

4.3.2 Optimization and validation of the mineral analysis

One of the advantages of the HR-CS-AAS is the potential to view the spectral environment of the analytical line at high resolution (M. Oliveira et al., 2012; S. R. Oliveira et al., 2010; Welz, 2005). A clear spectral environment was obtained, with no interference for the standard and samples solutions. The linearity range (R^2 > 0.995) was assessed using a least square fit (see Table 4.3). The analysis of iron, manganese and zinc was made directly from the digested solution, through fast sequential measurements of the three elements, reducing greatly the time of analysis when compared to the more conventional Linear Source equipment (LS-FAAS). Calcium and magnesium were

analysed, after dilution with ultrapure water, in the same solution through fast sequential measurements. Due to the high content of potassium and low linearity range recorded for sodium analysis (0.05-0.75 mg/L) (Table 4.3), these elements required a higher dilution of the sample digested solution. In potassium determination a lower optical path length was also used, to reduce the method sensitivity and avoid diluting the digested sample more than thousand times.

Instrumental LOD (0.004 µg/g – 0.438 µg/g) and LOQ (0.012 µg/g – 1.316 µg/g) for each mineral are presented in Table 4.3, and were adequate for the analysis of the samples. The RSD was lower than 4% for the intra-day precision and varied between 1.01 % and 11.15 % for the inter-day precision.

To assess the accuracy of the method, a certified reference material (BCR 679-white cabagge) was analysed. The certified values / obtained values for the micro-minerals present in the BCR 679 were: Iron: 55 ± 2.5 mg/kg / 54.61 ± 0.48 mg/kg; Manganese: 13.3 ± 0.5 mg/kg / 12.08 ± 0.16 mg/kg; Zinc: 79.7 ± 2.7 mg/kg / 79.99 ± 1.84 mg/kg; and Copper: 2.89 ± 0.12 mg/kg / 2.95 ± 0.47 mg/kg. These values represent as recovery percentage of 99.3%, 90.8%, 100.4% and 102.2%, respectively, confirming the accuracy of the method for the determination of these minerals in vegetable samples. Since no certified reference material was available to confirm the accuracy of the macro-minerals evaluation, a recovery study of post-digested spiked samples was performed at two fortification levels (Spike 1 and Spike 2) using six different vegetables, namely green lettuce, watercress, lamb's lettuce, wild rocket, spinach and parsley. The mean recovery percentages for the six samples are presented in table 4.3. All minerals showed good recovery values, ranging between 91.1% and 109.9%, confirming the method’s accuracy.

4.3.3 Baby leaf vegetables analysis

4.3.3.1 Mineral content and stability

The method described was applied to eight ready-to-eat baby leaf vegetables. The results obtained are summarized in Table 4.4. Organic wild rocket leaves and lettuce had the highest and lowest global mineral content, respectively (954.9 ± 18.6 mg/ 100 g fw; 414.9 ± 0.6 mg/100 g fw). The macro-minerals (K, Na, Ca, Mg and P) represent 99.7 ± 0.2 % of the mineral content determined, and potassium was the principal element found in all samples (51.9 ± 10.9 %). Calcium was the second mineral most common mineral with the exception of the swiss chard leaves which had a higher sodium content. The highest levels magnesium and phosphorus concentrations were found in the parsley leaves. In

most samples, iron was more than half of the total micro-mineral content determined and was the highest in parsley leaves.

The mineral content of the baby leaf samples showed the same profile described for more mature samples (Bozokalfa, Yağmur, Aşçıoğul & Eşiyok, 2011; Martins, 2010). Green vegetables are among the major dietary sources of potassium (Otten, Hellwig & Meyers, 2006), and the results showed that the baby leaf salads are no different. According to Miller-Cebert et al. (2009), Flyman and Afolayan (2008) and Khader and Rama (2003) maturity stage affects differently the content of the minerals present in the plant. Plant cells tend to accumulate potassium and exclude sodium. The resulting high K/Na ratios release potassium for essential metabolic functions such as stomatal opening (Flyman & Afolayan, 2008). Calcium tends to accumulate in more mature parts of the plant and high levels of phosphorous are related to initial development stages of vegetables (involved in the synthesis of new protoplasm) (Khader & Rama, 2003). Zinc assimilation is also related to the initial development stages of plant development, whilst iron is accumulated at more mature stages (Flyman & Afolayan, 2008). The baby leaf vegetables studied, with the exception of parsley leaves, showed higher calcium content than phosphorous, and iron was always higher than the zinc levels. Although maturity stage could affect the mineral content, there are relevant external factors, such as soil composition, pH, water availability, application of natural and artificial fertilizers, weather conditions and also differences among cultivars from the same species that could cause the differences found (Khader & Rama, 2003). Bioavailability of minerals is often discussed, because green vegetables are considered a good source of iron and zinc. The study presented by Chiplonkar et al. (1999) showed iron bioavailability higher in green raw vegetables than other type of vegetables or cereals. Flyman and Afolayan (2008) suggested that more immature leafy vegetables could be beneficial to address iron and zinc deficiencies. However, the actual bioavailability of iron or zinc at any maturity stages would be affected by interactions between them as well as other factors. Calcium bioavailability from vegetables is diminished in the presence of phytates, oxalates and high fibre levels, but is enhanced by the presence of citric and malic acids (Kawashima & Valente Soares, 2003). Based on this, baby leaf green vegetables could be a good source of calcium, since phytates and fibre are low in these products. In spinach or parsley, for example, calcium bioavailability will be decreased because of oxalates (Savage & Mårtensson, 2010).

Table 4.4 Mean content (± standard deviation) in mg/100 g of fresh weight of macro- and micro- minerals in baby leaf vegetables at the beginning and end of the storage period (day 1 and day 10). Asterisks indicate values statistically different ($p > 0.01$) between day 1 and day 10.

		K	Na	Ca	Mg	P	Fe	Mn	Zn	Cu
Green lettuce	*Day 1*	217.67± 10.16	48.14± 4.99	68.82± 2.18	34.23± 1.57	43.71± 7.69	1.46± 0.06	0.84± 0.04	0.39± 0.04	0.07± 0.00*
	Day 10	221.39± 7.78	44.31± 1.82	72.37± 3.11	34.04± 0.97	39.78± 0.00	1.40± 0.02	0.79± 0.03	0.39± 0.03	0.04± 0.00*
Swiss chard	*Day 1*	393.40± 5.08	119.85± 3.50	106.34± 5.33	77.29± 2.12	51.64± 3.30	0.66± 0.05*	0.42± 0.02	0.38± 0.02	0.06± 0.00
	Day 10	398.67± 9.14	120.57± 3.95	114.52± 2.82	80.40± 2.62	66.69± 2.14	0.56± 0.02*	0.41± 0.01	0.38± 0.01	0.06± 0.00
Watercress	*Day 1*	340.80± 15.78	43.66± 2.02	155.14± 11.99	28.19± 1.12	69.84± 0.89	0.63± 0.05	0.24± 0.02	0.34± 0.03	0.05± 0.00
	Day 10	343.51± 20.16	43.05± 1.01	159.54± 9.29	27.86± 0.88	84.15± 2.33	0.62± 0.03	0.23± 0.01	0.35± 0.01	0.05± 0.00
Lamb's lettuce	*Day 1*	463.02± 7.62	10.18± 0.82	96.05± 6.54	49.08± 1.49	75.53± 6.34	1.86± 0.07	0.38± 0.00	0.39± 0.02	0.14± 0.00
	Day 10	459.91± 8.70	9.94± 0.17	97.53± 0.29	51.33± 0.20	64.12± 4.12	1.82± 0.10	0.39± 0.00	0.40± 0.02	0.15± 0.00
Wild rocket	*Day 1*	426.30± 21.67	26.42± 1.12*	317.80± 18.32	50.23± 2.10	65.26± 2.32	0.91± 0.05*	0.22± 0.01	0.27± 0.02	0.06± 0.00*
	Day 10	419.26± 14.58	24.07± 0.93*	330.10± 17.64	47.01± 2.60	79.03± 0.74	0.72± 0.02*	0.20± 0.00	0.27± 0.00	0.05± 0.00*
Organic wild rocket	*Day 1*	504.85± 3.44	33.05± 0.35	275.50± 7.29*	77.65± 3.48	75.55± 1.41	0.97± 0.06	0.11± 0.00	0.33± 0.02	0.06± 0.00
	Day 10	503.65± 13.53	32.90± 1.77	252.67± 2.62*	71.09± 1.48	80.02± 0.17	0.93± 0.04	0.11± 0.01	0.33± 0.02	0.06± 0.00
Spinach	*Day 1*	521.30± 31.52	68.41± 1.72	88.39± 5.92	80.66± 3.71	73.33± 3.99	1.64± 0.08	0.59± 0.02	0.28± 0.02	0.11± 0.01
	Day 10	507.28± 23.55	71.39± 3.82	94.23± 1.87	82.48± 2.45	54.56± 3.86	1.67± 0.03	0.61± 0.02	0.31± 0.02	0.12± 0.01
Parsley	*Day 1*	224.96± 11.88	126.16± 3.67	184.55± 3.57	95.50± 1.29	185.90± 27.81*	2.78± 0.04	0.64± 0.03	0.91± 0.07	0.08± 0.00*
	Day 10	231.69± 14.00	124.75± 3.70	183.42± 2.92	95.86± 2.75	128.01± 2.66*	2.86± 0.17	0.62± 0.02	0.80± 0.04	0.08± 0.00*

All samples showed a stable mineral content, presenting in most cases no statistical differences ($p < 0.01$) between the first day and the last day of storage (see Table 4.4). Although, some significant differences ($p<0.01$) could be found (8 cases in the 72 analysed), corresponding to a small decrease (see Table 4.4). Since minerals are not metabolized during the storage period, the content should not alter significantly during shelf life (Sánchez-Mata, Cámara & Díez-Marqués, 2003). The differences could result from a slight decay in product quality due to microbial contamination at later stages of storage (Santos et al., 2011). Sánchez-Mata et al. (2003) found differences in the mineral content of green beans during the storage under different atmospheres.

4.3.3.2 Multivariate analysis

The mineral content of eight baby leaf was analysed by Linear Discriminant Analysis (LDA) to learn more about the differences between the mineral composition of the samples. The LDA analysis created a model that included the nine minerals analyzed, which were the variables described in table 4.5. The sodium was the first mineral selected in this model and, together with manganese, potassium and calcium, contributed most to the overall discrimination. A canonical analysis followed and the differences between the samples were displayed in a canonical variate scatterplot. All data were displayed graphically in three axes, corresponding to the first three canonical variates (root) that comprise 86.8% of the data information (Figure 4.1).

Table 4.5 Discriminant Function Analysis Summary (N of vars in model: 9; Wilks' Lambda: 0,000 approx. F (63,276)=777,03 p<0,0000)

	Partial - Lambda	**F-remove - (7,48)**	**p-level**	**Toler.**
Na	0.044	149.051	0.000	0.530
Mn	0.042	152.690	0.000	0.370
K	0.070	91.244	0.000	0.618
Ca	0.020	335.277	0.000	0.684
Mg	0.100	61.781	0.000	0.518
Cu	0.295	16.379	0.000	0.530
Zn	0.177	31.796	0.000	0.740
Fe	0.524	6.229	0.000	0.633
P	0.794	1.776	0.114	0.847

The first dimension (CV 1) represents 42.1% of the data variance, separating the samples according to their sodium, potassium and copper content. The second canonical

variate (CV 2) described 31% and the third (CV 3) 3.7%, where manganese and potassium were the principal variables, respectively, separating the samples. The relation amongst the variables is represented by:

CV1 = 1.01 [Na] – 0.58 [K] – 0.53 [Cu] – 0.30 [Ca] + 0.29 [Mg] -0.26 [Fe] + 0.19 [Mn] +0.06 [Zn] + 0.01 [P]

CV2 = 1.20 [Mn] -0.62 [Na] – 0.60 [Cu] – 0.59 [K] + 0.28 [Fe] – 0.06 [Ca] + 0.10 [Mg] – 0.07 [Zn]– 0.07 [P]

CV3 = – 0.83 [K] – 0.74 [Mn] + 0.59 [Ca] + 0.49 [Fe] + 0.36 [Na] + 0.34 [Mg] + 0.25 [Zn] + 0.24 [P] + 0.07 [Cu]

The swiss chard and parsley samples were separated from the others, mainly by their high sodium content, and were more closely related to the positive side of CV1. The lamb’s lettuce leaves showed some correlation with negative part of CV1, being distinguish by their high content of potassium and copper. The second dimension (CV2) helped to detect the differences between the mineral content of lettuce, separated from the other samples due to their highest manganese content. Parsley showed a distinct mineral profile being the only sample on the positive part of CV3, due to its high content of iron. Spinach leaves showed a close relationship with the negative part of this axis, due to its high potassium and manganese content. Wild rocket leaves from conventional and organic production were closely related to the same canonical variates, showing the different agricultural practices (conventional vs. organic) did not change drastically the mineral profile of these samples. Watercress and wild rocket leaves showed a similar mineral content which could be expected because of their phylogenetic relationship (they belong to Brassicaceae family).

4.4 Conclusion

The methodology proposed in this study to quantify macro- and micro minerals by HR-CS-AAS in leafy vegetable matrices was optimized and validated. The HR-CS-AAS method was simple and with less preparation steps than the conventional LS-AAS analysis due to the potential for rapid sequential measurements of different minerals. The optimized microwave digestion and analysis procedure were shown to be accurate and appropriate to determine the content of several minerals in different baby leaf vegetables, with recoveries ranging from 91 to 110%, suitable LOD and LOQ and appropriate intra-day and inter-day precisions.

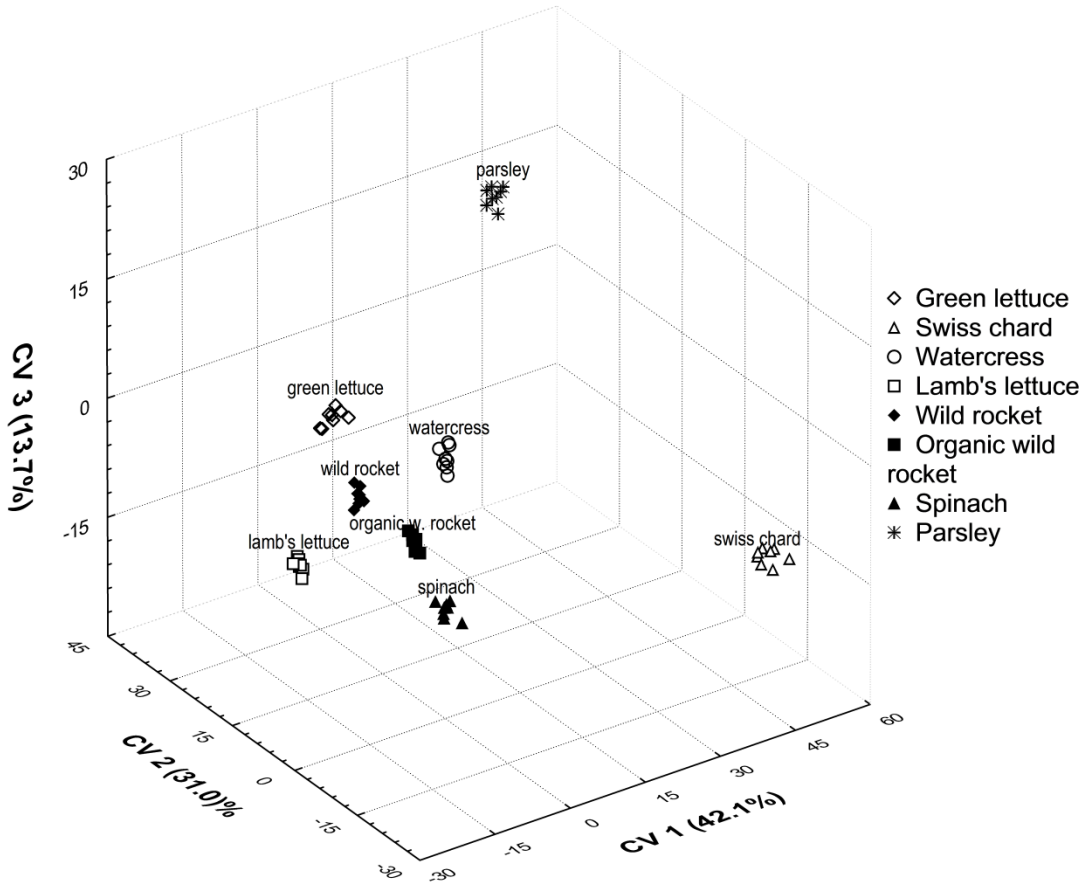

Figure 4.1 Plot of the first canonical variate (CV1) versus the second canonical variate (CV2) versus the third canonical variate (CV3) for the 8 baby leaf samples.

The baby leaf samples analysed showed a similar mineral profile as the more mature products, suggesting that these products could be a good source of these micronutrients. Their mineral content was stable through the 10 days of storage.

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CHAPTER 5.

Phenolic profile evolution of different ready-to-eat baby leaf vegetables during storage

This chapter presents the application of a pressurized liquid extraction method to extract phenolic compounds of 11 baby leaf samples that were identified and quantified by an HPLC-DAD-MS method. A comparison of the levels of phenolic compounds was made between the beginning and end of storage.

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Phenolic profile evolution of different ready-to-eat baby leaf vegetables during storage

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ABSTRACT

Ready-to-eat baby leaf vegetables market has been growing and offering to consumers convenient, healthy and appealing products, which may contain interesting bioactive compounds. In this work, the composition and the evolution of the phenolic compounds from different baby leaf vegetables during refrigerated storage was studied. The phenolic compounds were extracted using pressurized liquid extraction (PLE) and the phenolic profile of each sample was analysed and quantified by using LC-MS and LC-DAD methods, respectively, at the beginning and at the end of a 10-day storage period. The baby leaf vegetables studied included green lettuce, ruby red lettuce, swiss chard, spinach, pea shoots, watercress, garden cress, mizuna, red mustard, wild rocket and spearmint samples and a total of 203 phenolic compounds were tentatively identified and quantified. The main naturally phenolic compounds identified correspond to glycosylated flavonoids, with exception of green lettuce and spearmint leaves which had a higher content of hydroxycinnamic acids. Quantification of the main compounds showed a 10-fold higher content of total phenolic content of ruby red lettuce (483 mg g^{-1}) in relation to the other samples, being the lowest values found in the garden cress (12.8 mg g^{-1}) and wild rocket leaves (8.1 mg g^{-1}). The total phenolic content only showed a significant change ($p<0.05$) after storage in the green lettuce (+17.5%), mizuna (+7.8%), red mustard (-23.7%) and spearmint (-13.8%) leaves. Within the different classes of phenolic compounds monitored, the flavonols showed more stable contents than the hydroxycinnamic and hydroxybenzoic acids, although the behaviour of each compound varied strongly among samples.

Keywords: Baby leaf vegetables; PLE extraction; HPLC-DAD–MS; Phenolic compounds identification and quantification; Storage.

5.1 Introduction

The consumer demand for more convenient fresh food products led to a rapid grow of the fresh-cut industry, that became a multi-billion dollar sector worldwide in the last years. Fresh-cut vegetables can meet the consumer demands about the relationship between food, healthy lifestyle and convenience [1]. They are elaborated without additives, by minimal processing methods such as washing, cutting and packaging at chilling temperatures with polymeric films. Baby leaf salads have gained popularity over the traditional fresh-cut salads, by adding more variety to the diet and offering a product that attracts consumers and producers. The baby leaves are mixed, washed and packaged as whole, maintaining an appealing 3-D structure, reduced oxidation damage due to a small stem diameter and greater stability during shelf life [2-4]. Lettuces, rocket, watercress, spinach, and mustard greens are among the most used baby leaves, being sold individually or in salad mixtures [3].

The consumption of fresh vegetables is encouraged, not only due to their micronutrient composition (normally rich in vitamins and minerals), but also due to their phytochemicals, that are believed to protect human health [5]. Some antioxidant, anti-inflammatory and antitumor effects have been attributed to certain phytochemicals, that are also related to the vegetable color and flavor [5,6]. Within the European Union there is no specific regulation related to the presence of phytochemicals, but any nutrition and/ or health claim made on the labels must be based on scientific studies that take into consideration the composition of phytochemicals and their qualitative and quantitative characteristics [5,7]. The antioxidant properties of the vegetables are one of the most present label claims due to the high levels of carotenoids, tocopherols and ascorbic acid that have epidemiological evidence of beneficing human health [6]. In the other hand, the antioxidant properties of vegetable intake are also closely related to the presence of phenolic compounds. These are secondary metabolites of the plants, characterized by having at least one aromatic ring with one or more hydroxyl groups [6,8]. Polyphenols can range from simple molecules (phenolic acids) to more complex structures (e.g., phenylpropanoids or flavonoids) or even highly polymerized compounds (such as lignins or tannins), with flavonoids representing the most common and widely distributed sub-group [9]. Moreover, different types and numbers of sugars and functional derivatives such as esters or methyl esters can be conjugated to aglycones, forming numerous structures of phytochemicals, being described more than 8000 natural phenolic compounds [6,8,10]. The phenolic content of a plant is affected by several factors like plant species, cultivar, environmental conditions, water availability, light exposure,

germination, maturity, processing and storage [5,11]. In minimally processed fresh-cut products, the shredding step can increase the antioxidant capacity associated with wound-induced phenolic compounds [12]. Reyes, et al. [13] described major changes in the total soluble phenolic content during the storage of fresh-cut vegetables, influenced by the initial levels of reduced ascorbic acid and phenolic compounds. Also light exposure and temperature of storage can induced the synthesis of certain phenolic compounds.

The importance of phenolic compounds as potential antioxidants and their complex chemical structure, variability and distribution creates a challenge to properly assess their content in food products. Traditionally, techniques to extract phenolic compounds from fresh or freeze-dried vegetables use large amounts of hydro-organic solvent mixtures [14] and are normally very laborious, time-consuming and not very selective. Pressurized Liquid Extraction (PLE) has been shown to be a more environment friendly alternative to extract bioactive compounds from a vegetal matrix [15]. PLE combines elevated temperature and pressure with the use of minimum amounts of food-grade solvents to achieve a fast and efficient extraction of several compounds, while preserving their bioactivity and chemical structure. A better diffusion of the solvent into the matrix is obtained by maintaining the pressures and temperatures below the critical point of the solvents, due to a higher solubility of the analytes in the solvent and to the decrease of solvent viscosity and surface tension [15]. PLE has been successfully applied to the extraction of phenolic compounds from vegetable matrices, showing high yields, better recoveries, being more time efficient and economic when compared to the traditional methods [14,16,17].

As the popularity of ready-to-eat baby leaf vegetables increases, there is an urgent need to understand how the profile of important components of these more immature vegetables evolves during storage. In this sense, it has been already demonstrated how some fat- and water-soluble free vitamin losses may be produced during refrigerated storage of these products [18]. The purpose of this work was to study the evolution of the phenolic compounds of a wide group of ready-to-eat baby leaf vegetables during storage, including green lettuce, ruby red lettuce, swiss chard, spinach, watercress, garden cress, mizuna, red mustard, wild rocket, peashoots and spearmint. To do that, a PLE method was optimized to extract the phenolic compounds from these vegetables and the obtained extracts were analyzed by HPLC–DAD–MS. The information available so far focuses more on the identification of the phenolic compounds present in these baby leaves [19-22]. Moreover, there are only a few publications on the changes of the phenolic compound during the storage of baby leaf samples, mainly for spinach leaves [23-25], wild rocket [26] and lettuces [27].

5.2 Materials and Methods

5.2.1 Chemicals and standard solutions

Methanol was of HPLC-grade and acquired from LabScan (Dublin, Ireland) whereas ethanol was purchased from Scharlab (Barcelona, Spain). Folin–Ciocalteau phenol reagent and sodium carbonate (Na_2CO_3) were acquired from Merck (Darmstadt, Germany) and the water used was Milli-Q Water (Millipore, Billerica, MA, USA). Formic acid, gallic acid, 4- hydroxybenzoic acid, sinapic acid, syringic acid, caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid, quercetin, kaempferol and catechin were supplied by Sigma–Aldrich (Madrid, Spain). The others phenolic standards, i.e. vanillic acid, rosmaniric acid, quercetin-3-rutinoside, quercetin-3-rhamnoside, quercetin-3-glucoside, quercetin-3-galactoside, luteolin-7-glucoside, apigenin-7-glucoside, kaempferol-3-glucoside, apigenin and diosmetin were acquired from Extrasynthese (Genay, France).

Individual phenolic standard solutions were prepared in 70% MeOH solution with the following concentrations: 0.2 mg mL^{-1} for caffeic acid, p-coumaric acid, quercetin, apigenin, apigenin-7-glucoside and kaempferol-3-glucoside; 0.5 mg mL^{-1} for syringic acid; 0.7 mg mL^{-1} for luteolin-7-glucoside, kaempferol and diosmetin; 1.0 mg mL^{-1} for sinapinic acid, rosmarinic acid, salicylic acid, p-hydroxybenzoic acid, vanillic acid, gallic acid, quercetin-3- rhamnoside, quercetin-3-rutinoside and catechin; and 1.4 mg mL^{-1} for chlorogenic acid, ferulic acid, quercetin-3-galactoside and quercetin-3-0-glucoside. All standard solutions were kept under refrigeration at 4 ºC until analysis. During development of HPLC-DAD-MS method, a mixture of phenolic standards was prepared by dilution of the individual phenolic stock solutions with 70% MeOH solution.

5.2.2 Samples

Minimally processed baby leaf vegetables, washed and packaged, were supplied by a producer (Odemira, Portugal) of minimally processed vegetables. The samples were received in the laboratory one day after being processed, individually packaged and in the same conditions normally used for distribution and commercialization of fresh-cut products. The samples comprised 4 of the most common baby leaves used in ready-to eat salads, namely, green lettuce, ruby red lettuce, swiss chard and spinach, 5 baby leaf vegetables from the Brassicaceae family characterized by their peppery flavor, namely, watercress, garden cress, mizuna, red mustard and wild rocket, a baby leaf recently

introduced in salad mixtures, pea shoots, and a fresh-cut aromatic herb that can be added to ready-to-eat salads, such as spearmint. Each sample was divided into two batches, corresponding to the two sampling times studied (day 1 and day 10). After sampling, the baby leaf samples were freeze-dried (Telstar Cryodos-80, Terrassa, Spain) until analysis. The ten-day refrigerated storage (3 ± 1 ºC) period was monitored with an EL-USB 2 (Lascar Electronics, Salisbury, UK). The freeze-dried leaves were reduced to a fine powder in a knife mill (GM 200, Retsch, Haan, Germany) and stored protected from light, oxygen and high temperatures. This procedure intended to exclude individual differences and ensure the representativeness of the test sample.

5.2.3 Pressurized Liquid Extraction (PLE)

The extractions were performed using an accelerated solvent extraction system (ASE 200, Dionex, Sunnyvale, CA) equipped with a solvent controller. The PLE method was first optimized using pea shoots and spearmint as test samples. Methanol/water and ethanol/water mixtures in three different proportions (50%, 70% and 90%) were the solvents tested to extract phenolic compounds from freeze dried vegetable leaves. The extraction temperature (70ºC), pressure (10 MPa), flush volume (60% of cell volume using extraction solvent), sample quantity (0.5 g) and dispersion (2 g of sea sand) were maintained constant during this study. The static extraction time was of 20 min, including an additional 5 min heat-up step prior to any extraction. After choosing the solvents, different durations for the static extraction and number of cycles of extraction were tested in 5 different combinations: i) 10 min × 1 extraction cycle; ii) 20 min × 1 extraction cycle; iii) 30 min × 1 extraction cycle; iv) 5 min × 2 extraction cycles; v) 10 min × 2 extraction cycles. The extraction procedure was performed as previously described [16]. All the extractions were done using 11 mL extraction cells and, between extractions, a rinse of the complete system was performed to avoid any carry-over. The extracts were protected from light and stored under refrigeration until dried. A Rotavapor R-210 (Büchi, Labortechnik AG, Flawil, Switzerland) was used to evaporate the organic solvents from the extracts. The remaining aqueous phase was freeze-dried (Labconco Corporation, Missouri, USA) to obtain a completely dried extract. Dried extracts were re-dissolved in 70% MeOH (5 mg mL^{-1}) and filtered through a 0.45µm disposable syringe filter. All extractions were made by duplicate.

5.2.4 Determination of total phenols content (Folin–Ciocalteu method)

The total phenols content of the different PLE extracts was determined by using the Folin–Ciocalteu assay [28] with some small changes [29], and was expressed as mg gallic acid equivalents/g dry extract. Briefly, 10 µL of sample (5 mg mL^{-1} in 70% MeOH) and 50 µL of undiluted Folin–Ciocalteu reagent were added to 600 µL of water. After 1 minute, 150 µL of 20% (w/v) Na_2CO_3 was added to the mixture and, finally, the volume was made up to 1 mL with deionized water. This reaction mixture was incubated for 2 h at 25 ºC and then, 300 µL were transferred to a well of a 96-well microplate. Absorbance was measured at 760 nm in a microplate spectrophotometer reader Powerwave XS (BioTek Instruments, Winooski, VT) and compared to a gallic acid calibration curve (linear range: 0.015–1 mg/mL, R^2>0.997).

5.2.5 Analysis of phenolic compounds by HPLC-DAD-MS

The phenolic compounds present in the PLE extracts of the baby leaf samples were analyzed by HPLC-DAD-ESI-MS^n on an Agilent 1200 liquid chromatograph (Agilent, Santa Clara, CA, USA) equipped with an autosampler, a DAD, and directly coupled to an ion trap mass spectrometer (Agilent ion trap 6320) via an electrospray interface. The column used was a Zorbax Eclipse XBD C_{18} (5 µm, 150×4.6 mm) (Agilent, Santa Clara, CA) and the mobile phases were water (0.1% formic acid, A) and MeOH (0.1% formic acid, B). The gradient employed was the following: 0 min, 95% A; 4 min, 95% A; 20 min, 73% A; 50 min, 5% A; 57 min, 99% A; 58 min, 99% A; 60 min, 95% A. A flow rate of 0.7 mL min^{-1} was used together with an injection volume of 10 µL. The diode array detector recorded the spectra from 200 to 550 nm, being each run monitored at 280, 330 and 370 nm. The MS detector operated under ESI negative ionization mode using the following parameters: dry temperature, 350 ºC; dry gas flow, 12 L min^{-1}; nebulizer gas pressure, 40 psi; capillary voltage, 3500 V. The instrument mass scan range was *m/z* 100 to 1000 and MS/MS automatic mode was used on the more abundant ions in the MS spectra to study their fragmentation patterns.

The phenolic compounds were identified by comparing their retention time, UV-Vis and mass spectra with those obtained from standard solutions, when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature. To identify the presence of acyl flavonoid derivatives on the samples, an alkaline hydrolysis was carried out to eliminate acid

moieties (e.g., p-coumaroyl, caffeoyl, feruloyl and sinapoyl), following the procedure described by Francisco, et al. [30].

For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of other compound from the same phenolic group. The results were expressed in μg g^{-1} of dry weight (d.w.), as mean ± standard deviation of two extracts. At least, two replicates of each extract were made for quantification purposes.

5.2.6 Statistical analysis

The results were expressed as mean ± standard deviation and the differences of phenolic contents between the two sampling days (day 1 and day 10) were tested using one-way ANOVA test. Differences were considered as statistically significant at a value of $p < 0.05$. The statistical analyses were carried out using Statistica 8.0 software (Statsoft Inc., Tulsa, USA).

5.3 Results and Discussion

As mentioned, the phenolic profile of a vegetable can be influenced by intrinsic factors related to the plant development stage and genetic variability. Also, some external factors will affect the plants' phenolic metabolism in response to agronomic and environmental conditions, post-harvesting processing and also storage conditions. Therefore, the phenolic profile of baby leaf vegetables will be the result of the interaction of both intrinsic and external factors on their development. In a previous work, it was already demonstrated how the amount of free vitamins in these products decreased during storage [18]. In the present work the same analysis procedure was applied to all samples in order to study the stability of another important group of bioactive-relevant compounds, polyphenols. It included an optimization of a PLE method, followed by the development of the HPLC method to separate, identify and quantify the phenolic compounds present. The concentration of the individual phenolic compounds was compared between the samples collected in the beginning and at the end of the storage period.

5.3.1 Samples extraction

In this work, one of the objectives was to use a “green” extraction method of phenolic compounds that could be applied to different samples of baby leaf vegetables. In order to optimize the PLE conditions to extract phenolic compounds from the studied vegetables, the total phenols content of the extracts was monitored (Figure 5.1).

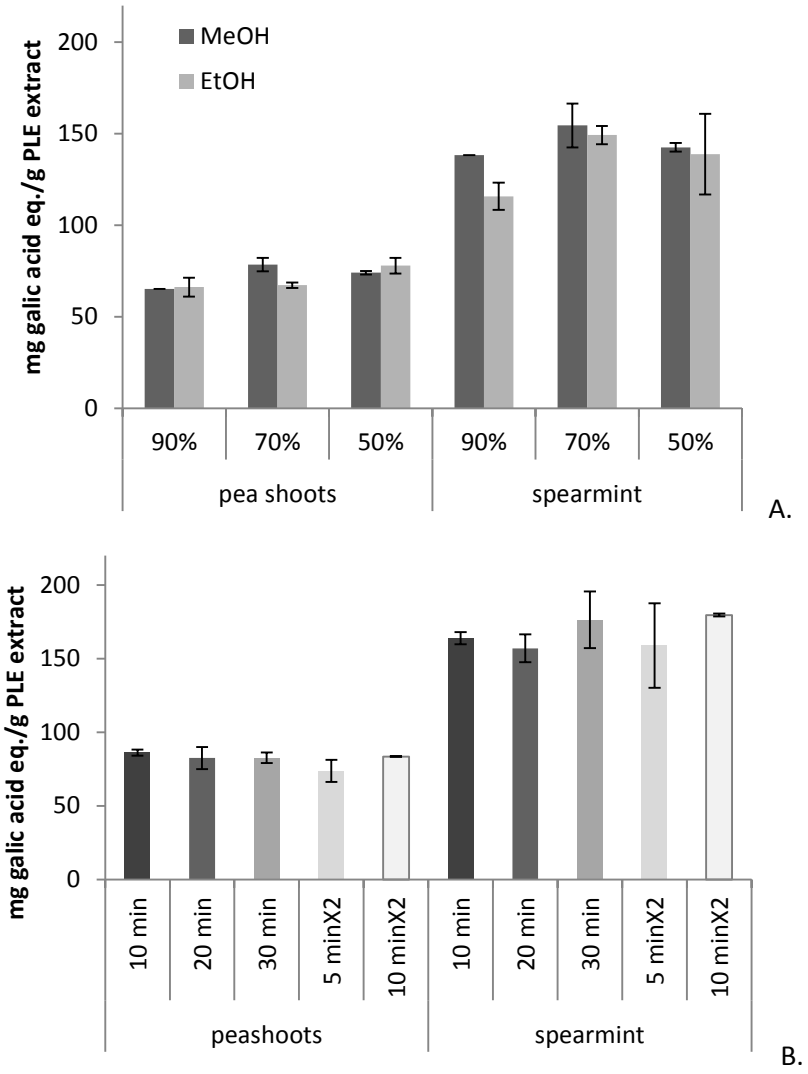

Figure 5.1 Total phenols content obtained with PLE of the two samples at the indicated conditions (A. Solvents mixtures comparison; B. static extraction time and number of extraction cycles).

First of all, the influence of using different combinations of hydro-organic solutions with MeOH or EtOH was tested. As can be observed in Figure 5.1A, there were no statistically significant differences ($p<0.05$) between the use of MeOH or EtOH in the extractions for a particular proportion in the two different vegetables considered as models (pea shoots and spearmint), showing similar values for each concentration of organic solvent. The use of

90% of organic solvent revealed a lower yield of total phenols, compared to the use of 70% and 50%. These latter proportions produced similar results, although the use of 70 % of organic solvent slightly improved the obtained results, and between them, MeOH allowed the attainment of a higher total phenols value (5% and 7% more in pea shoots and spearmint, respectively). These results were similar to those obtained in the PLE of parsley flakes [31]. Consequently, 70% MeOH in water was the solvent chosen to perform the study about the influence of the extraction time and number of extraction cycles.

Regarding the extraction time, no significant differences ($p<0.05$) were revealed at the different studied extraction times for both vegetables (Figure 5.1B). These results are in agreement with previous PLE studies, where the time of extraction also showed a minor influence on the total extraction yield and total antioxidant activity of the extracts [32,33]. Spearmint PLE extracts obtained with 2 extraction cycles of 10 min had a 12% higher phenol content than the extract produced with a single static extraction of 20 min. The use of 2 static cycles of 10 min also produced, in both samples, the smaller variation between replicates (<1%). Even though, a static extraction cycle of 10 min produced similar yield than the longer extractions, the use of 2-ten min cycles was selected due to better results obtained for spearmint and considering that this process could be more appropriate to cover the potential variability that might exist among samples [15,31].

Concerning the rest of the extraction conditions, the influence of the extraction pressure has been described to have a negligible effect on the overall extraction yield once it is enough to maintain the solvent in the liquid state [15,17]. Thus, 10 MPa was set as extraction pressure. On the other hand, temperature is considered to have a major influence on the extraction yield. Normally, high temperatures improved the diffusion rate of the analytes of interest to the solvent, but it can also promote the co-extraction of other compounds, decreasing the selectivity of the extraction [17]. The use of very high temperatures can also affect the stability of more thermo-labile compounds and also may drive to the formation of new compounds [34]. A temperature of 70 ºC was chosen based on other published works [30,35], in order to improve the transfer rate of the analytes to the solvent while maintaining the natural phenolic profile present in the baby leaves, avoiding the formation of other compounds.

5.3.2 Phenolic profile of ready-to-eat baby leaf vegetables

5.3.2.1 Characterization of the PLE baby leaf extracts

In first place, a new HPLC method was optimized to achieve the best separation of the 23 phenolic compounds for which commercial standards were available (see Figure 5.2 and Table 5.1 for peak identification). These compounds were preliminarily chosen according to the phenolic composition described in the literature for some of the studied species [6,14,19,21]. Most compounds were correctly separated with very good resolution; however, the separation of quercetin-3-*0*-rutinoside and quercetin-3-*0*-glucoside (peak 14+15) was not achieved, co-eluting in the conditions selected (Figure 5.2). The chromatograms corresponding to the studied samples are also presented in Figure 5.2. Each baby leaf PLE extract showed a particular and distinctive phenolic profile, with a good separation of the compounds. For each phenolic profile the identification of the compounds was attempted by combining the information of the DAD and the MS detector, together with retention times and information available on the literature. A preliminary analysis of the compounds' UV-Vis spectra allowed the classification of the separated peaks into two classes of phenolic acids (namely, hydroxycinnamic and hydroxybenzoic acid derivatives) and into three classes of flavonoids (flava-3-ol, flavonol and flavone derivatives). Hydroxybenzoic acids and flava-3-ols were detected at 280 nm, whereas hydroxycinnamic acids exhibited an absorbance maximum around 320-330 nm, flavonols between 350 and 385 nm, and flavones in the 277–295 nm range with a shoulder at 300–330 nm [36]. Due to the fact that in the nature, polyphenols occur conjugated to sugars and organic acids, the comparison between the UV-Vis spectra and retention time with the available standards did not permit the complete identification of most of compounds present in the samples. The study of the hydrolyzed extracts, comparing the native and the hydrolyzed profile, permitted the identification of the acyl glycosylated compounds that include in their structure an acid moiety; those compounds were not present in the hydrolyzed extracts, appearing with more intensity the hydroxycinnamic acids (caffeic, p-coumaric, ferulic and sinapic acids) that would be present in a conjugated form in the native extracts, and also other flavonoids mainly conjugated with sugar moieties (see Figure 5.3). Combining the information obtained from the UV-Vis spectra obtained from the native and hydrolyzed extracts together with the analysis of the MS spectra and fragmentation patterns of the main ions detected, the separated compounds could be tentatively assigned on the different samples.

Figure 5.2 HPLD-DAD chromatograms (280 nm) of the phenolic standards and PLE baby leaf extracts of all samples. For peak identification and information see Tables 5.1, 5.2 and 5.3.

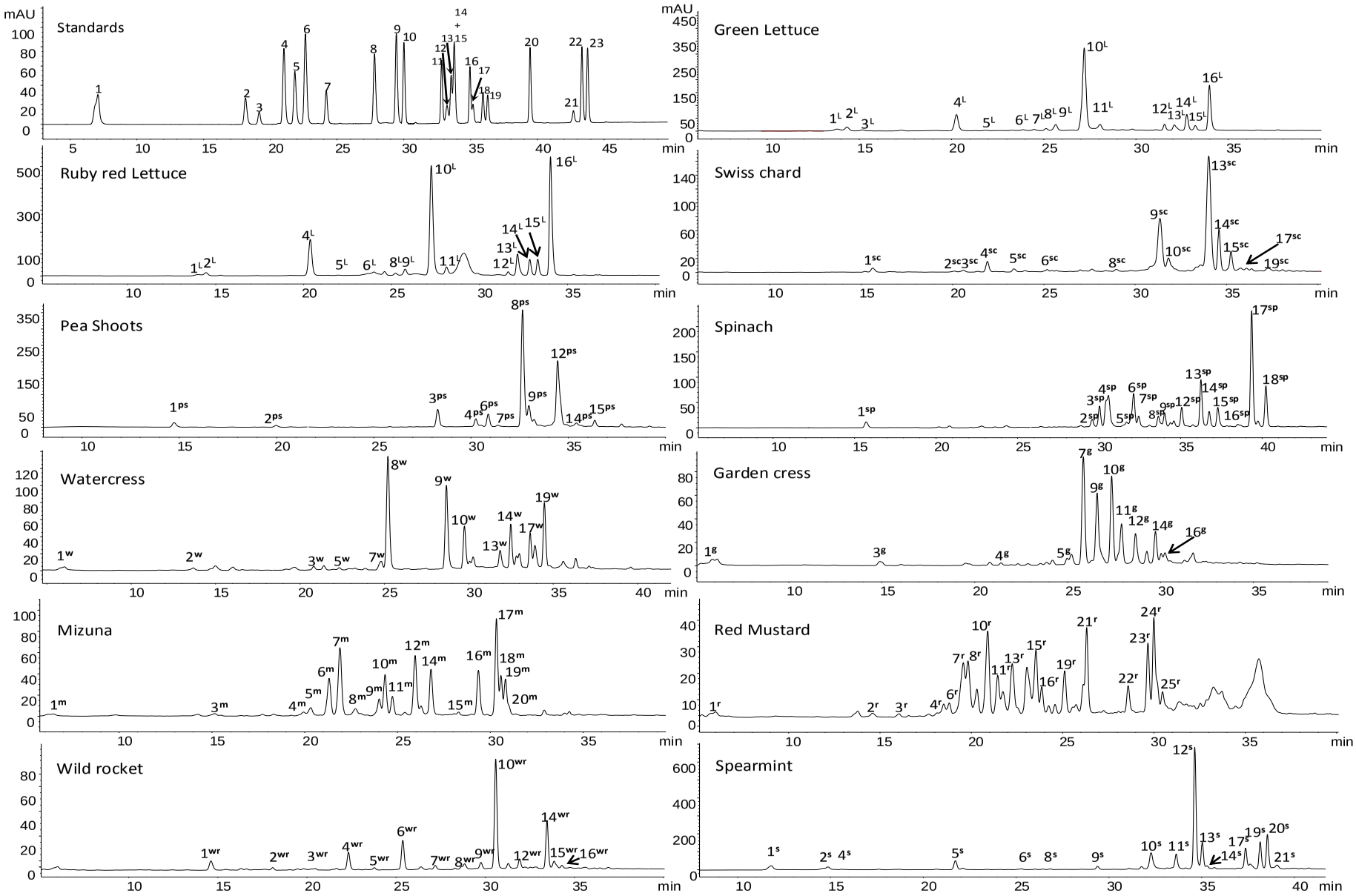

Table 5.1 Phenolic standards identification and retention time, linearity, limits of detection (LOD), limits of quantification (LOQ) and repeatability obtained with the HPLC–DAD method (Sh, spectral shoulder).

		Detection				LOD	LOQ	Linearity		Repeatability (RSD %)			
		RT (min)	UV–Vis maxima (nm)	$[M-H]^-$ (m/z)	MS/MS (main fragments)	µg/ml	µg/ml	µg/ml	R^2	INTRA-DAY (n=5)		INTER-DAY (n = 15)	
Peak	**Name**									RT	Area	RT	Area
1	Gallic acid	6.3	271	169	125	0.08	0.23	0.2- 47.6	0.999	1.0	0.9	1.2	1.8
2	p-hydroxybenzoic acid	16.9	256	137	93	0.16	0.48	0.5-52.4	0.999	0.5	1.6	0.7	1.8
3	Catechin	18.1	280	289	245; 205; 179	0.40	1.21	1.2-47.6	0.999	0.4	1.5	0.5	1.9
4	Chlorogenic acid	20.0	242; 298 sh; 326.	353	191; 179	0.15	0.46	0.5-50.0	0.998	0.9	1.1	0.9	3.3
5	Vanillic acid	20.7	260; 292	167	152; 123; 108	0.14	0.44	0.4-73.3	0.998	1.0	1.2	1.0	3.2
6	Caffeic acid	21.4	238; 298 sh; 323	179	135	0.09	0.28	0.3-47.5	0.998	1.1	0.9	1.1	3.1
7	Syringic acid	23.2	274	197	179; 135	0.11	0.33	0.3-35.7	0.999	0.3	1.7	0.4	2.0
8	p- Coumaric acid	26.6	228; 310	163	119	0.11	0.32	0.3-45.0	0.997	0.9	1.2	0.9	3.4
9	Ferulic acid	28.5	236; 298 sh; 322	193	179; 135	0.08	0.23	0.2-66.7	0.999	0.3	1.6	0.4	1.9
10	Sinapic acid	29.0	238; 323	223	208; 179; 164	0.10	0.31	0.3-60.5	0.999	0.2	1.5	0.3	1.8
11	Luteolin-7-0-glucoside	32.0	256; 268 sh; 350	447	285	0.22	0.67	0.7-34.9	0.999	0.2	1.3	0.3	1.6
12	Salicylic acid	32.1	236; 302	137	93	0.41	1.25	1.3-80.0	0.999	0.7	1.2	0.8	2.3
13	Quercetin-3-D-galactoside	32.7	256; 268 sh; 356	463	301	0.09	0.26	0.3-66.7	0.998	0.3	1.4	0.3	1.8
14	Quercetin-3-*O*-rutinoside	32.9	256; 268 sh; 356	609	301	0.07	0.20	0.2-73.3	0.998	0.5	1.1	0.5	3.5
15	Quercetin-3-*O*-glucoside	33.0	256; 268 sh; 356	431	301	0.08	0.24	0.2-66.7	0.999	0.2	1.5	0.3	1.9
16	Rosmarinic acid	34.3	330; 290 sh	359	161	0.21	0.63	0.6-440.0	0.997	0.4	1.0	0.4	3.3
17	Apigenin-7-*O*-glucoside	34.5	268; 336	431	269	0.20	0.62	0.6-11.9	0.999	0.2	2.8	0.2	2.5
18	Quercetin-7-*O*-rhamnoside	35.2	256; sh268; 356	447	301	0.10	0.32	0.3-57.1	0.999	0.2	1.4	0.2	1.8
19	Kaempferol-3-*O*-glucoside	35.5	266; 348	447	285	0.07	0.20	0.2-14.8	0.999	0.2	1.3	0.3	1.7
20	Quercetin	38.8	256; 372	301		0.05	0.15	0.2-47.6	0.999	0.2	0.9	0.2	0.9
21	Kaempferol	42.0	266; 338	285		0.04	0.12	0.1-37.0	0.999	0.2	1.3	0.2	1.5
22	Apigenin	42.7	268;338	269		0.05	0.15	0.2-11.9	0.999	0.2	1.5	0.2	1.8
23	Diosmetin	43.1	252; sh270; 346	299		0.14	0.43	0.4-28.6	0.999	0.2	1.4	0.2	1.8

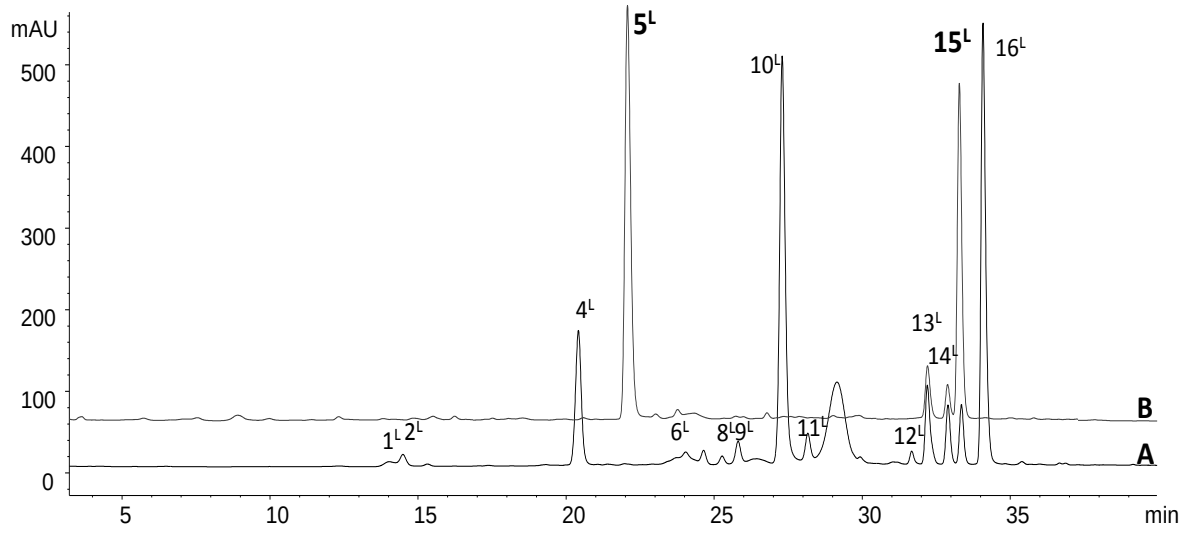

Figure 5.3 HPLC-DAD (280 nm) chromatogram of ruby red lettuce phenolic profile. A, native extract and B, hydrolyzed extract. (For peak identification see Table 5.2).

In Tables 5.2 and 5.3 the identification of the major phenolic compounds of each sample is presented. In some cases, the low concentration of the compound in the extract, or the high background noise in the MS signal did not permit to clearly identify the main ions of the compound or the fragmentation pattern. In those cases the compounds identification was based only on their UV-Vis spectra and was referred as a derivative of the more similar aglycone. Thorough MS analysis of pure compounds could theoretically make possible to assign the positional isomers of the glycosylated flavonoids. However, in the present work, when it was not possible to unambiguously characterize those flavonoids, the most frequently found form, containing a 7-*O*-linkage, was assumed.

In total, 203 compounds from the 11 baby leaf samples were tentatively identified. All the samples showed particular chemical compositions with the exception of two baby leaf lettuces (green and ruby red) which presented a similar phenolic profile (Table 5.2). Chlorogenic acid (4^{L}: 5-caffeoylquinic acid) and chicoric acid (10^{L}) were the two major hydroxycinnamic acids present in these samples, that were also composed by several quercetin derivatives and a flavone (luteolin-7-*O*-glucuronide, peak 13^{L}). These baby leaf samples reveal the same composition that other works described for different lettuce samples [36,37]. Flavonoids (9^{sc}-19^{sc}) were the main compounds present in swiss chard. Peaks 9^{sc}, 10^{sc}, 12^{sc} and 13^{sc} corresponded to the flavone apigenin, conjugated with sugar moieties, identified by the presence of a ion at m/z 269 $[M-H]^-$ in the MS^2 experiments (Table 5.2). These compounds were described as characteristic in the phenolic composition of swiss chard leaves [39]. Quercetin (peaks 11^{sc}, 17^{sc} and 18^{sc}) (MS^2 ion at

m/z 301 [M-H]$^-$) and isorhamnetin (peaks 14^{sc}, 15^{sc} and 19^{sc}) (MS^2 ion at m/z 315[M-H]$^-$) derivatives where also identified (Table 5.2).

Spinach leaves showed a great number of flavonols, especially patuletin (MS^2 ion at m/z 331[M-H]$^-$) and spinacetin (MS^2 ion at m/z 345[M-H]$^-$) derivatives (peaks 3^{sp}, 5 ^{sp}-18^{sp}), described in the literature as the main phenolic compounds of these leaves [14,23,24,41]. Flavonols were also the main compounds found in pea shoots (peaks 3^{ps} to 15^{ps}). To our knowledge, the phenolic compounds of pea leaves had only been studied in a previous work [42] in which 7 flavonols were identified. In the present work, 5 of the previously referred compounds were detected (peaks 3^{ps}, 4^{ps}, 6^{ps}, 8^{ps} and 9^{ps}), as well as other compounds that were identified for the first time in this baby leaf product, corresponding to peaks 10^{ps} to 15^{ps} (Table 5.2). In pea shoots, the presence of hydroxycinnamic acids was not verified as individual compounds, although their presence was confirmed as conjugates with flavonols (6^{ps}, 8^{ps}, 9^{ps}, 12^{ps}, 13^{ps} and 15^{ps}), thanks to the detection of p-coumaric, sinapic and ferulic acids in its corresponding hydrolyzed extract.

Watercress, garden cress, mizuna, red mustard and wild rocket are all plants belonging to the Brassicaceae family, which includes numerous economically important species that had been widely investigated in terms of glucosinolates and phenolic composition [6,21,30]. In these samples, flavonols were the main group of phenolic compounds, showing different quercetin, kaempferol and isorhamnetin derivatives (Table 5.3). As could be expected, these samples showed some common phenolic compounds between them. For instance, the presence of ferulic (peaks 10^{w}, 14^{g}, 17^{m}, 23^{r}) and sinapic (11^{w}, 15^{g}, 18^{m}, 24^{r}) acids derivatives appeared in watercress, garden cress, mizuna and red mustard. Concerning the flavonoid content, 9 matching compounds were found in the phenolic profile of mizuna and red mustard leaves, corresponding to isorhamnetin 3-O-glucoside-7-O-glucoside (14^{m}, 21^{r}), kaempferol-3-(methoxycaffeoyl-diglucoside)-7-glucoside (6^{m}, 10^{r}), kaempferol 3-(caffeoyl-diglucoside)-7-glucoside (7^{m}, 11^{r}), kaempferol-3-(sinapoyl-diglucoside)-7-glucoside (9^{m}, 15^{r}), kaempferol- 3-(feruloyl-diglucoside)-7-glucoside (10^{m}, 16^{r}), kaempferol-3-(p-coumaroyl-diglucoside)-7-glucoside (11^{m}, 17^{r}), quercetin 3-hydroxyferuloylsophoroside-7-glucoside (4^{m}, 7^{r}), quercetin-3-caffeoyldiglucoside-7-glucoside (5^{m}, 8^{r}) and quercetin-3-(sinapoyl-diglucoside)-7-glucoside (8^{m}, 13^{r}).

Table 5.2 Retention time (Rt), UV-Vis maxima, mass spectral data, tentative identification and concentration of phenolic acids and flavonoids in the baby leaf PLE extracts of green lettuce, ruby red lettuce, swiss chard, spinach and pea shoots. (* Significant differences ($p<0.05$) between day 1 and day 10; # [2M−H]−; n.d. not detected).

Peak	Rt	λ max (nn)	$[M - H]^-$	MS^2	ref.	Tentative identification	Quantification	
							Day 1	Day 10
							mg.g^{-1} (d.w) ± sd	***mg.g^{-1} (d.w) ± sd***
Green Lettuce								
1[L]	14.3	242;sh 298;328	311	179, 149, 135	[36]	Caftaric acid	367.2 ± 30.6	466.8 ± 139.0
2[L]	14.7	240;sh 298;328	623[#]	491, 311	[36]	Caftaric acid *	633.2 ± 50.2	824.6 ± 186.6
3[L]	15.5	278				Hydroxybenzoic acid derivative*	264.7 ± 11.5	571.9 ± 149.2
4[L]	20.5	242;sh 298;326	353	191, 179	[36]	Chlorogenic acid	3165.9 ± 14.2	3750.1 ± 886.7
5[L]	21.8	236;sh 298;324				Caffeic acid*	9.3 ± 4.4	71.5 ± 19.4
6[L]	24.1	256;sh268;350	625	463, 301	[37]	Quercetin- di- glucoside *	137.6 ± 18.7	339.7 ± 94.3
7[L]	24.7	256;sh 266; 354	725	681, 505	[37]	Quercetin-3-malonylGlucoside-7-glucuronide	224.5 ± 44.6	202.1 ± 68.6
8[L]	25.3	256;sh268; 354	711	667	[37]	Quercetin-3-malonylGlucoside-7-glucoside *	305.8 ± 60.4	486.7 ± 121.3
9[L]	25.9	244;sh 298;328	295	179	[37]	Caffeoylmalic acid *	713.7 ± 41.9	1073.6 ± 277.0
10[L]	27.5	244; sh 298; 328	473	311,179, 149	[36]	Chicoric acid	11687.8 ± 244.0	14708.1 ± 3439.1
11[L]	28.3	244; sh 298;330	473	311,179, 149	[36]	Dicaffeoyltartaric acid isomer *	769.4 ± 12.0	1144.1 ± 262.8
12[L]	31.7	242;sh 298; 242	515	353,179	[36]	Di-*O*-caffeoylquinic acid *	700.9 ± 28.8	1273.2 ± 360.4
13[L]	32.3	254;sh 266;350	461	285	[37]	Luteolin-7-*O*-glucuronide	980.7 ± 45.6	984.0 ± 240.0
14[L]	32.9	256;sh 268; 354	477	301	[36]	Quercetin-3-glucuronide	3125.5 ± 40.7	3396.2 ± 783.8
15[L]	33.3	256;sh 268;354	463	301	[36]	Quercetin-3-*O*-glucoside	1244.5 ± 437.7	1466.6 ± 428.6
16[L]	34.1	256;sh 268; 354	549	505,463,301	[36]	Quercetin-3-malonylglucoside	9960.2 ± 418.7	10482.7 ± 2390.2
						Hydroxycinnamic acids*	*17 786.4 ± 405.5*	*23 121.1 ± 5 550.4*
						Hydroxybenzoic acids *	*264.7 ± 11.5*	*571.9 ± 149.2*
						Flavones	*980.7 ± 45.6*	*984.0 ± 240.0*
						Flavonols	*15 199.0 ± 255.5*	*16 818.0 ± 3 973.3*
						Total polyphenols*	*34 131.4 ± 458.9*	*41 399.7 ± 9 762.1*

Table 5.2. Continued (* Significant differences (p<0.05) between day 1 and day 10; # [2M−H]−; n.d. not detected).

Peak	Rt	λ max (nn)	$[M - H]^-$	MS^2	ref.	Tentative identification	Quantification	
							Day 1	Day 10
							mg.g^{-1} (d.w) ± sd	***mg.g^{-1} (d.w) ± sd***
Ruby Red Lettuce								
1^L	14.4	242;sh 298;328	311	179, 149, 135	[36]	Caftaric acid	1588.7 ± 207.6	1483.1 ± 136.4
2^L	14.9	240;sh 298;328	623#	491, 311	[36]	Caftaric acid *	4095.8 ± 964.7	3977.7 ± 590.5
3^L	15.7	278				Hydroxybenzoic acid derivative	2600.2 ± 172.6	2414.3 ± 281.1
4^L	20.6	242;sh 298;326	353	191, 179	[36]	Chlorogenic acid*	48054.6 ± 7376.9	29572.2 ± 1173.6
5^L	21.5	236;sh 298;324				Caffeic acid*	<LOQ	387.9 ± 53.4
6^L	23.8	256;sh268;350	625	463, 301	[37]	Quercetin 3,7-di-*O*-glucoside	8535.7 ± 1032.1	8451.4 ± 683.1
8^L	25.4	256,sh 266;354	711	667	[37]	Quercetin-3-malonylglucoside-7-glucoside	2452.0 ± 310.2	2712.6 ± 360.9
9^L	26.1	244,sh 298;328	295	179	[37]	Caffeoylmalic acid*	4909.9 ± 975.1	2269.7 ± 405.2
10^L	27.7	244;sh 298;328	473	311,179, 149	[36]	Chicoric acid*	100706.5 ± 9056.1	81625.6 ± 4198.5
11^L	28.5	244;sh 298; 330	473	311,179, 149	[36]	Dicaffeoyltartaric acid isomer *	7765.0 ± 538.0	5943.3 ± 435.6
12^L	31.8	242;sh 298; 328	515	353,179	[36]	Di-*O*-caffeoylquinic acid *	3470.2 ± 364.6	4071.7 ± 440.6
13^L	32.4	254;sh 268; 348	461	285	[37]	Luteolin-7-*O*-glucuronide*	28587.2 ± 2982.0	33088.1 ± 2669.3
14^L	33.1	256;sh 268; 354	477	301	[36]	Quercetin-3-glucuronide	26980.5 ± 2221.3	29529.4 ± 2008.0
15^L	33.5	256;sh 268; 354	463	301	[36]	Quercetin-3-*O*-glucoside	26685.5 ± 11706.1	40561.3 ± 19428.7
16^L	34.2	256;sh 268; 354	549	505,463,301	[37]	Quercetin-3-malonylglucoside	223483.6 ± 15908.7	233806.5 ± 21649.5
						Hydroxycinnamic acids*	*170 342.8 ± 17 882.6*	*127 716.9 ± 6 021.6*
						Hydroxybenzoic acids	*1 093.6 ± 70.5*	*1 019.6 ± 112.1*
						Flavones*	*28 587.2 ± 2 982.0*	*33 088.1 ± 2669.3*
						Flavonols	*285 819.5 ± 26 819.3*	*315 185.7 ± 22 768.6*
						Total polyphenols	*482 913.3 ± 34 158.2*	*477 010.1 ± 30 984.6*

Table 5.2. Continued (* Significant differences (p<0.05) between day 1 and day 10; # [2M−H]−; n.d. not detected).

Peak	Rt	λ max (nn)	$[M - H]^-$	MS^2	ref.	Tentative identification	Quantification	
							Day 1	Day 10
							mg.g^{-1} (d.w) ± sd	***mg.g^{-1} (d.w) ± sd***
Swiss chard								
1 sc	15.4	280				Catechin derivative *	597.4 ± 65.3	1209.8 ± 165.0
2 sc	19.9	228, 316				P- coumaric acid derivative*	2.0 ± 1.7	7.0 ± 2.0
3 sc	20.3	284				Syringic acid derivative	68.9 ± 10.8	79.6 ± 12.0
4 sc	21.7	240,sh 298, 330	353	173,191	[38]	4-caffeoylquinic acid *	529.5 ± 50.9	901.6 ± 111.9
5 sc	23.2	239, 322				Sinapic acid derivative	66.7 ± 5.9	76.3 ± 9.9
6 sc	25.0	241,sh298, 324	529	459,193	[38]	Caffeoylferuloylquinic acid	46.4 ± 5.7	55.1 ± 7.7
7 sc	27.8	244,sh 298, 328				Caffeic acid derivative*	54.5 ± 23.4	2.9 ± 0.4
8 sc	29.2	242,sh 298, 322				Caffeic acid derivative*	19.7 ± 3.6	7.9 ± 1.9
9 sc	31.2	268,338	563	413, 293	[39]	Apigenin-2''-*O*-pentoxide-8-*C*-hexoside (Vitexin 2''-xyloside)	4257.8 ± 649.6	4636.3 ± 694.7
10 sc	31.7	268,338	605	334,293	[39]	Apigenin acetylapiosylglucoside	888.5 ± 98.6	976.9 ± 134.4
11 sc	32.9	256,sh 268, 352	595	477,301	[39]	Quercetin pentosylhexoside	261.4 ± 54.0	180.2 ± 26.2
12 sc	33.5	270,336				Apigenin derivative*	476.5 ± 75.3	353.2 ± 43.6
13 sc	33.9	270,335	649	564,403	[39]	Apigenin malonylapiosylglucoside (Vitexin 6''-*O*-malonyl-2''-*O*-xyloside)	6526.0 ± 589.5	7144.3 ± 968.4
14 sc	34.5	254, sh 268, 356	639	315	[40]	Isorhamnetin-diglucoside	2680.3 ± 207.8	2740.3 ± 394.3
15 sc	35.2	254,sh 268, 354	609	315	[40]	Isorhamnetin-3-pentaside-7-glucoside	1300.4 ± 107.3	1414.2 ± 197.2
16 sc	35.7	270,324				Apigenin derivative	218.9 ± 17.2	231.1 ± 31.8
17 sc	36.0	256,sh 268, 334	801	625,301	[40]	Quercetin-3-feruloylsophoroside*	211.8 ± 34.9	297.5 ± 44.5
18 sc	36.3	256,sh 268,352				Quercetin derivative *	170.8 ± 21.6	130.2 ± 18.4
19 sc	37.2	270,330	623	461,315		Isorhamnetin-3-caffeoyl -7- rhamnosyl *	217.1 ± 32.1	158.7 ± 20.4
						Hydroxycinnamic acids	*9 956.6 ± 799.7*	*10 985.6 ± 1 476.2*
						Hydroxybenzoic acids	*68.9 ± 10.8*	*79.6 ± 12.0*
						Flavones	*12 367.8 ± 1 002.0*	*13 341.7 ± 1 810.7*
						Flavonols	*4 736.4 ± 479.1*	*4 954.1 ± 704.4*
						Flavan-3-ols*	*597.4 ± 65.3*	*1 209.8 ± 165.0*
						Total polyphenols	*27 064.3 ± 2 181.0*	*29 328.0 ± 3 983.4*

Table 5.2. Continued (* Significant differences (p<0.05) between day 1 and day 10; # [2M−H]−; n.d. not detected).

Peak	Rt	λ max (nn)	[M - H]⁻	MS²	ref.	Tentative identification	Quantification	
							Day 1	Day 10
							mg.g⁻¹ (d.w) ± sd	***mg.g⁻¹ (d.w) ± sd***
Spinach								
1 sp	15.7	278				Gallic acid derivative*	219.9 ± 9.2	296.2 ± 12.2
2 sp	29.6	312				P- coumaric acid derivative	157.7 ± 12.5	156.4 ± 13.2
3 sp	30.0	258;sh 272; 351	797	655,331	[24]	Patuletin-3-glucosyl-(1-6)[apiosyl 1-2)]-glucoside	1327.7 ± 64.2	1329.0 ± 52.1
4 sp	30.6	230; 314	338	163	[38]	3-*O*-p-coumaroylquinic acid	1477.5 ± 107.4	1422.2 ± 87.2
5 sp	31.7	258;sh 270; 347	933	787,769,658,331	[41]	Spinacetin-3-*O*-β-D-glucopyranosyl-(1 → 6)-[β-*D*-apiofuranosyl-(1 → 2)]-β-*D*-glucopyranoside	394.5 ± 56.5	361.4 ± 65.7
6 sp	32.1	256;sh 270; 352	801	655,345	[24]	Spinacetin-3-glucosyl-(1-6)[apiosyl 1-2)]-glucoside	2547.7 ± 120.5	2584.2 ± 90.8
7 sp	32.4	250; sh 272; 335	963	787,801	[41]	Patuletin-3-*O*-β-D-(2''-feruloylglucopyranosyl)-(1 → 6)-[β-D-apiofuranosyl-(1 → 2)]-β-D-glucopyranoside	889.9 ± 56.5	939.0 ± 29.8
8 sp	33.6	258; 274;318	948	701,345	[41]	Spinacetin-3-*O*-β-D-(2''-ρ-coumaroylglucopyranosyl-(1 → 6)-[β-apiofuranosyl-(1 → 2)]-β-D-glucopyranoside	751.3 ± 34.2	754.8 ± 27.8
9 sp	34.0	250; 274; 336	977	783, 673, 345	[41]	Spinacetin-3-(2′′-feruloylglucosyl)(1-6)[apiosyl 1-2)]-glucoside	1111.4 ± 59.7	1147.9 ± 39.6
10 sp	34.4	316	801	658	[24]	Patuletin with gentiobioside and rhamnoside	225.5 ± 10.3	230.6 ± 7.5
11 sp	34.6	333	831	655	[41]	Patuletin-3-*O*-β-D-(2''-feruloylglucopyranosyl)-(1 → 6)-β-D-glucopyranoside	473.0 ± 19.8	498.2 ± 7.3
12 sp	35.1	256; sh 272; 352	669	345	[41]	Spinacetin-3-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside	1362.3 ± 53.2	1379.3 ± 48.1
13 sp	36.3	270; 340	521	345	[24]	Spinacetin glucuronide	2880.3 ± 125.6	2997.4 ± 78.4
14 sp	36.8	253; 332	845	669, 499, 345	[41]	Spinacetin-3-*O*-β-D-(2''-feruloylglucopyranosyl)-(1 → 6)-β-D- glucopyranoside)*	1198.7 ± 49.8	1276.5 ± 21.1
15 sp	37.3	272; 340	535	359	[24]	Jaceidin 4-glucuronide	1210.1 ± 61.5	1240.1 ± 40.7
16 sp	38.5	258; 313; 358	845	669, 499, 345	[41]	Spinacetin-3-*O*-β-D-(2''-feruloylglucopyranosyl)-(1 → 6)-β-D-glucopyranoside	185.4 ± 9.0	196.4 ± 7.0
17 sp	39.4	278; 336	519	345	[41]	5, 3,4-trihydroxy-3-methoxy-6:7-methylenedioxyflavone 4-glucuronide	6813.1 ± 261.4	6855.6 ± 121.5
18 sp	40.2	278; 340	533	345	[41]	5,4-dihydroxy-3,3-dimethoxy-6:7-methylenedioxyflavone 4-glucuronide	2386.7 ± 93.6	2332.9 ± 53.0
						Hydroxycinnamic acids	*1 635.2 ± 119.9*	*1 578.5 ± 100.3*
						Hydroxybenzoic acids*	*219.9 ± 9.2*	*296.2 ± 12.2*
						Flavonols	*24 189.6 ± 1102.2*	*24 540.4 ± 634.7*
						Total polyphenols	*26 044.7 ± 1231.3*	*26 415.1 ± 743.9*

Table 5.2. Continued (* Significant differences (p<0.05) between day 1 and day 10; # [2M−H]−; n.d. not detected).

Peak	Rt	λ max (nn)	[M - H]⁻	MS²	ref.	Tentative identification	Quantification	
							Day 1	Day 10
							mg.g⁻¹ (d.w) ± sd	***mg.g⁻¹ (d.w) ± sd***
Pea shoots								
1 ps	15.2	278				Hydroxybenzoic acid derivative*	313.1 ± 6.4	619.4 ± 13.0
2 ps	20.6	282				Hydroxybenzoic acid derivative*	120.8 ± 5.8	153.1 ± 7.9
3 ps	28.8	256;sh 268; 352	787	667,625,445,301	[42]	Quercetin-3-*O*-sophorotrioside*	2831.2 ± 31.7	2717.7 ± 24.7
4 ps	30.7	266; 347	771	651,609,429,285	[42]	Kaempferol-3-*O*-sophorotrioside*	889.3 ± 34.6	816.9 ± 31.6
5 ps	31.0	266; 346	771	651,609,429,285	[42]	Kaempferol-3-*O*-sophorotrioside*	145.4 ± 9.9	123.7 ± 14.2
6 ps	31.3	252;sh 268;332	949	787	[21]	Quercetin-3-(caffeoyl-diglucoside)-7-glucoside*	2088.4 ± 47.9	2267.6 ± 85.5
7 ps	31.8	268; 318				Kaempferol derivative	251.2 ± 52.1	203.0 ± 36.3
8 ps	33.1	226; 316	933	787	[21]	Quercetin-3-(p-coumaroyl-diglucoside)-7-glucoside	16329.0 ± 127.3	16384.7 ± 292.8
9 ps	33.3	251;sh 268; 331	963	787		Quercetin- 3-feruloylsophoroside-7-glucoside*	3735.6 ± 73.6	3972.3 ± 90.5
10 ps	33.6	256;sh268; 354	963	787		Quercetin-3-*O*-(glucuronide-diglucoside)-7-glucoside	987.2 ± 17.3	1023.6 ± 47.4
11 ps	34.1	266; 336	979	771	[40]	Kaempferol-3-sinapoylsophotrioside	593.3 ± 74.1	519.8 ± 66.8
12 ps	34.9	268; 316	917	771	[40]	Kaempferol-3-(p-coumaroyl-diglucoside)-7-glucoside	9114.2 ± 114.4	9102.2 ± 413.2
13 ps	35.3	268; 320	947	771,753,609,285	[40]	Kaempferol-3-feruloylsophoroside-7-glucoside	291.4 ± 23.0	379.7 ± 97.1
14 ps	35.8	266; 349	933	771	[40]	Kaempferol-3-*O*-sophorotrioside-7-glucoside	738.1 ± 67.3	732.2 ± 50.2
15 ps	36.7	267; 315	917	771	[40]	Kaempferol-3-(p-coumaroyl-diglucoside)-7-glucoside	714.9 ± 70.8	678.0 ± 34.4
						Hydroxybenzoic acids*	*409.8 ± 49.0*	*772.5 ± 16.0*
						Flavonols	*38 789.6 ± 403.2*	*38 988.9 ± 1116.7*
						Total polyphenols	*39 199.4 ± 413.8*	*39 761.4 ± 1119.1*

Phenolic profile evolution

In the PLE extract of watercress, different quercetin-related compounds were identified (peaks 6^{w}, 7^{w}, 13^{w} and 14^{w}) [21] as well as different isorhamnetin derivatives (peaks 16^{w}, 18^{w}). Watercress phenolic profile also showed a significant presence of hydroxycinnamic derivatives (peaks 4^{w}, 5^{w} and 9^{w}-12^{w}), being mainly caffeic, p-coumaric, sinapic and ferulic acids derivatives (Table 5.3).

In garden cress leaves, the flavonols found correspond to quercetin (peaks 5^{g} and 6^{g}) and kaempferol (peaks 7^{g} -12^{g}) conjugated with glucoside, rhamnosyl, caffeoyl, p-coumaroyl and synapoyl. Also the presence of sinapic (peaks 4^{g}, 13^{g} and 15^{g}) and ferulic acid (14^{g} and 16^{g}) derivatives was detected. These were the same type of compounds that were also described in the study of the phenolic composition of garden cress sprouts [45]. Mizuna and red mustard leaves showed a flavonoid composition similar to the one described in the literature for those species [21,40]. Red mustard PLE extracts revealed the most complex profile among the studied samples in terms of number of compounds present (Figure 5.2). A total of 25 phenolic compounds were tentatively identified; as can be seen in the complete profile obtained (Figure 5.2), kaempferol, quercetin and isorhamnetin glycosides were the main compounds of this vegetable (6^{r} to18^{r} and 20^{r} to 21^{r}), mainly conjugated with ferulic, sinapic and p-coumaric acid. In the case of wild rocket leaves, the MS analysis only permitted the complete identification of 6 compounds (2^{wr}, 5^{wr}, 6^{wr}, 8^{wr}, 10^{wr} and 11^{wr}), being the other classified accordingly to the UV-Vis spectra and complemented with the data obtained from the analysis of the hydrolyzed extract.

Lastly, spearmint is an aromatic herb from the Lamiaceae family that includes plants recognized as being a good source of phenolic compounds, like oregano and rosemary [16,46]. The compounds found in spearmint were mainly caffeic acid derivatives, like chlorogenic (4^{s}), rosmaniric (12^{s}), lithospermic (13^{s}) and salvionic (17^{s}) acids. Besides the rosmaniric acid (molecular ion at m/z 359$[M-H]^{-}$), that was the main compound of the spearmint phenolic profile, 5 compounds were identified as rosmaniric acid derivatives (15^{s} and 19^{s}-22^{s}), due to the presence of a fragmentation ion at 359$[M-H]^{-}$.

Table 5.3 Retention time (Rt), UV-Vis maxima, mass spectral data, tentative identification and concentration of phenolic acids and flavonoids in the baby leaf PLE extracts of watercress, garden cress, mizuna, red mustard, wild rocket and spearmint. (* Significant differences ($p<0.05$) between day 1 and day 10; # [2M−H]−; n.d. not detected).

Peak	Rt	λ max (nn)	$[M - H]^-$	MS/MS	ref.	Tentative identification	Quantification	
							Day 1	Day 10
Watercress							***mg.g^{-1} (d.w) ± sd***	***mg.g^{-1} (d.w) ± sd***
1 ^{w}	6.3	274				Hydroxybenzoic acid derivative	307.6 ± 42.4	280.6 ±18.5
2 ^{w}	14.0	280				Catechin derivative *	280.9 ± 13.2	478.1 ±17.6
3 ^{w}	21.1	224; 310				Gallic acid derivative	170.3 ± 20.7	197.3 ± 41.8
4 ^{w}	21.7	240;sh 300;328				Caffeic acid derivative*	114.0 ± 6.9	55.4 ± 5.0
5 ^{w}	22.6	240; 332				Sinapic acid derivative*	92.8 ± 5.1	37.5 ± 0.2
6 ^{w}	24.2	258;sh268; 354	625	463,505,346,301	[40]	Quercetin-7-*O*-sophoroside	80.3 ± 12.2	68.2 ± 1.5
7 ^{w}	25.1	254;sh268; 342	771	609, 301	[43]	Quercetin-3-*O*-rutinoside-7-*O*-glucoside*	447.5 ± 62.8	335.6 ± 14.1
8 ^{w}	25.6	242;sh298; 328	591$^{\#}$	295, 179	[37]	Caffeoylmalic acid acid*	2983.9 ± 196.2	2731.0 ± 70.1
9 ^{w}	29.1	228; 312	581 163	465, 301 119	[40]	P-coumaric acid derivative	1361.2 ± 99.7	1416.1 ±33.5
10 ^{w}	30.1	240;sh 298; 328	193	149, 178,134	[40]	Ferulic acid derivative*	859.0 ± 63.7	960.7 ± 25.2
11 ^{w}	30.3	242; 330				Sinapic acid derivative	370.8 ± 23.0	395.6 ±11.9
12 ^{w}	30.6	236; 324				Caffeic acid derivative	240.3 ± 45.5	226.6 ±42.9
13 ^{w}	32.1	252;sh 268; 338	933	787, 625, 301	[21]	Quercetin-3-(caffeoyl- diglucoside)-7-rhamnosyl	834.9 ± 40.2	966.3 ±137.1
14 ^{w}	32.7	254;sh 268; 338	873	829		Quercetin-3-caffeoylglucoside-6''-malonylglucoside	3202.0 ± 680.8	2597.2 ±110.9
15 ^{w}	33.1	242; 330	737	529	[40]	Disinapoylgentiobiose	465.2 ± 76.9	512.6 ± 38.5
16 ^{w}	33.1	249;sh 270; 342	977	831, 669, 609, 315	[40]	Isorhamnetin-3-hydroxyferuloylglucoside-7-glucoside	987.9 ± 142.3	939.8 ± 153.2
17 ^{w}	33.9	247;sh 268; 338	917	873,609,285		Non-identified flavonol	1904.4 ± 163.0	1851.0 ± 67.5
18 ^{w}	34.1	254;sh 268; 333	947	639,609,315		Isorhamnetin-3-(caffeoyl-diGlucoside)-7-Rhamnosyl	1594.7 ± 341.7	1519.7 ± 58.9
19 ^{w}	34.7	254;sh 268; 332	887	843		Non-identified flavonol	3513.2 ± 329.7	3260.8 ± 262.9
						Hydroxycinnamic acids	*6 591.0 ± 505.1*	*6 504.1 ± 168.4*
						Hydroxybenzoic acids	*477.9 ± 57.3*	*477.9 ± 50.8*
						Flavonols	*13 697.5 ± 1396.0*	*12 692.6 ± 652.2*
						Flavan-3-ols *	*280.9 ± 13.2*	*478.1 ± 17.6*
						Total polyphenols	*21 047.3 ± 1 900.4*	*20 152.7 ± 830.0*

Table 5.3**. Continued** (* Significant differences (p<0.05) between day 1 and day 10; # [2M−H]−; n.d. not detected).

Peak	Rt	λ max (nn)	[M - H]$^-$	MS/MS	ref.	Tentative identification	Quantification	
							Day 1	Day 10
Garden cress							***mg.g^{-1} (d.w) ± sd***	***mg.g^{-1} (d.w) ± sd***
1 g	5.9	262				Hydroxybenzoic acid derivative	170.1 ± 17.2	177.8 ± 5.5
2 g	6.1	272	647	323	[44]	Tri-*O*-galloylquinic acid	127.5 ± 9.2	133.7 ± 8.8
3 g	14.9	278				Gallic acid derivative*	99.4 ± 3.3	570.6 ± 9.0
4 g	22.3	244; 332				Sinapic acid derivative*	71.1 ± 2.6	19.7 ± 0.7
5 g	24.2	252;sh 268; 338	933	787, 771, 625	[21]	Quercetin- 3-(p-coumaroyl-diglucoside)-7-glucoside *	170.8 ± 20.9	217.0 ± 15.0
6 g	25.0	234;sh 268; 332	977	831, 771, 625	[21]	Quercetin 3-(sinapoyl-diglucoside)-7-rhamnosyl*	223.7 ± 16.1	191.8 ± 18.6
7 g	25.2	244; 330	917	771	[21]	Kaempferol 3-(caffeoyl-diglucoside)-7-rhamnosyl *	500.6 ± 32.7	400.8 ± 16.3
8 g	25.8	268; 332	917	771	[21]	Kaempferol 3-(caffeoyl-diglucoside)-7-rhamnosyl *	3146.2 ± 109.7	2958.5 ± 34.2
9 g	26.3	270; 318	917	771	[21]	Kaempferol 3-(p-coumaroyl –diglucoside)-7-rhamnosyl	105.5 ± 14.0	115.5 ± 7.1
10 g	26.6	228; 268; 333	961	815, 755, 609	[40]	Kaemferol-3-p-coumaroylsinapoyl-diglucoside	2905.9 ± 118.6	2847.5 ± 16.0
11 g	27.4	268; 332	931	785	[45]	Kaempferol-feruloylglucoside-diglucoside	2497.5 ± 95.3	2565.6 ± 17.2
12 g	27.9	268; 318	901	755,609		Kaempferol- 3-p-coumaroylglucoside-diglucoside	1331.1 ± 74.6	1398.6 ± 24.1
13 g	28.6	226; 316				Sinapic acid derivative*	451.3 ± 27.1	493.0 ± 13.6
14 g	29.7	236; 327	193	179,135		Ferulic acid*	447.5 ± 22.5	387.5 ± 1.5
15 g	30.0	240;329	223	163		Sinapic acid *	412.3 ± 28.2	319.5 ± 1.6
16 g	30.2	236;324				Ferulic acid derivative*	169.0 ± 9.1	221.8 ± 4.6
						Hydroxycinnamic acids*	*1551.2 ± 83.7*	*1441.5 ± 9.3*
						Hydroxybenzoic acids*	*397.0 ± 22.7*	*882.1 ± 6.7*
						Flavonols	*10 881.3 ± 457.0*	*10 695.3 ± 86.7*
						Total polyphenols	*12 829.5 ± 559.6*	*13 018.9 ± 90.3*

Table 5.3. Continued (* Significant differences (p<0.05) between day 1 and day 10; # [2M−H]−; n.d. not detected).

Peak	Rt	λ max (nn)	[M - H]$^-$	MS/MS	ref.	Tentative identification	Quantification	
							Day 1	Day 10
Mizuna							***mg.g^{-1} (d.w) ± sd***	***mg.g^{-1} (d.w) ± sd***
1 m	6.36	272	647	323	[44]	Tri-*O*-galloylquinic acid *	175.8 ± 10.5	128.6 ± 4.5
2 m	14.9	242; sh 298; 324				Caffeic acid derivative	36.9 ± 1.5	38.5 ± 4.7
3 m	15.3	280				Gallic acid derivative*	n.d.	513.4 ± 4.3
4 m	19.8	250; sh 268; 334	979	817, 787, 625	[40]	Quercetin 3-(methoxycaffeoyl-diglucoside)-7-glucoside	245.3 ± 5.8	246.1 ± 6.5
5 m	20.1	253;sh 268; 335	949	787,625	[40]	Quercetin-3-(caffeoyl-diglucoside)-7-glucoside	372.7 ± 19.1	348.9 ± 59.0
6 m	21.1	268; 331	963	801	[40]	Kaempferol-3-(methoxycaffeoyl-diglucoside)-7-glucoside	1725.3 ± 21.2	1751.2 ± 112.5
7 m	21.7	268; 331	933	771	[21]	Kaempferol 3-(caffeoyl-diglucoside)-7-glucoside *	2666.4 ± 35.3	2365.1 ± 166.1
8 m	22.6	250; sh 268; 340	993	831,787,625		Quercetin-3-(sinapoyl-diglucoside)-7-glucoside		
			963	801,625	[21]	Quercetin- 3-(feruloyl-diglucoside)-7-glucoside	494.0 ± 7.6	437.0 ± 64.2
			933	787,771,625		Quercetin- 3-(p-coumaroyl-diglucoside)-7-glucoside		
9 m	23.8	268; 334	977	815	[21]	Kaempferol- 3-(sinapoyl-diglucoside)-7-glucoside*	728.5 ± 10.3	826.7 ± 64.8
10 m	24.2	268; 332	947	785	[21]	Kaempferol- 3-(feruloyl-diglucoside)-7-glucoside	1387.5 ± 16.8	1501.3 ± 95.8
11 m	24.5	268; 318	917	755	[40]	Kaempferol-3-(p-coumaroyl-diglucoside)-7-glucoside	538.6 ± 6.3	506.8 ± 37.1
12 m	25.7	242;sh 298; 328	613	497, 317, 201		Caffeic acid derivative	1240.9 ± 19.7	1286.5 ± 95.1
13 m	26.1	266; 342	609	477, 285	[40]	Kaempferol-3-*O*-glucoside-7-*O*-glucoside	324.1 ± 1.6	337.0 ± 25.0
			933	771		Kaempferol-3-*O*-triglucoside-7-*O*-glucoside		
14 m	26.6	252;sh265; 340	639	477	[21]	Isorhamnetin-3-*O*-glucoside-7-*O*-glucoside*	2735.6 ± 34.2	3131.6 ± 197.2
15 m	27.9	268; 314	917	755	[41]	Kaempferol-3-(p-coumaroyl-diglucoside)-7-glucoside	117.2 ± 14.2	109.2 ± 9.6
16 m	29.1	226; 312	581	465, 301, 163		P-coumaric acid derivative*	549.0 ± 7.0	644.3 ± 43.8
17 m	30.1	238;sh 298; 328	641	525		Ferulic acid derivative*	1499.1 ± 21.6	1744.5 ± 112.7
			193	178,149, 134				
18 m	30.4	240; 329	701	585		Sinapic acid derivative *	1669.6 ± 23.3	2125.2 ± 218.3
			223	208,179,164				
19 m	30.6	324				Ferulic acid derivative	425.5 ± 9.4	435.4 ±47.8
20 m	30.8	324				Ferulic acid derivative *	144.1 ± 6.8	112.0 ± 6.5
						Hydroxycinnamic acids*	*5 585.61 ± 78.1*	*6 291.81 ± 377.1*
						Hydroxybenzoic acids*	*175.81 ± 10.5*	*642.01 ± 5.0*
						Flavonols	*11 335.01 ± 132.2*	*11 560.81 ± 818.5*
						Total polyphenols*	*17 045.61 ± 269.1*	*18 494.61 ± 1191.7*

Table 5.3. Continued (* Significant differences (p<0.05) between day 1 and day 10; # [2M−H]−; n.d. not detected).

Peak	Rt	λ max (nn)	[M - H]⁻	MS/MS	ref.	Tentative identification	Quantification	
							Day 1	Day 10
Red Mustard							***mg.g⁻¹ (d.w) ± sd***	***mg.g⁻¹ (d.w) ± sd***
1 r	5.8	260				Hydroxybenzoic acid derivative*	55.1 ± 5.2	38.8 ± 2.9
2 r	14.2	279				Gallic acid derivative*	49.9 ± 4.3	259.2 ± 6.7
3 r	15.7	244;sh 298; 328				Caffeic acid derivative*	11.8 ± 1.9	n.d.
4 r	17.4	232; 328				Sinapic acid derivative*	33.6 ± 2.2	n.d.
5 r	18.2	252;sh 268; 340				Flavonol*	366.1 ± 23.1	221.2 ± 11.5
6 r	18.5	254;sh 268; 337	639	477		Isorhamnetin-3-caffeoyl-7-glucoside	218.1 ± 19.7	230.8 ± 10.1
7 r	19.3	246;sh 265; 338	979	817, 787, 625	[40]	Quercetin-3-(methoxycaffeoyl-diglucoside)-7-glucoside*	1668.9 ± 82.6	945.5 ± 51.7
8 r	19.5	252,sh268, 336	949	787, 625	[40]	Quercetin-3-(caffeoyl-diglucoside)-7-glucoside*	1250.4 ± 84.2	911.1 ± 33.5
9 r	20.0	270; 332				Flavonol*	545.9 ± 39.8	450.0 ± 20.6
10 r	20.6	268; 330	963	801,771, 609	[21]	Kaempferol-3-(methoxycaffeoyl-diglucoside)-7-glucoside*	1839.6 ± 84.4	1023.3 ± 41.6
11 r	21.1	268; 330	933	771	[21]	Kaempferol-3-(caffeoyl-diglucoside)-7-glucoside *	722.8 ± 30.6	487.4 ± 22.4
12 r	21.4	250;sh 268;338				Non-identified flavonol	433.8 ± 21.4	448.1 ± 21.2
13 r	22.0	248;sh 268; 338	993	831, 787, 625		Quercetin-3-(sinapoyl-diglucoside)-7-glucoside*	1509.6 ± 71.4	1266.6 ± 58.7
14 r	22.8	242;sh270; 334				Non-identified flavonol	1485.3 ±76.5	1409.9 ± 64.2
15 r	23.3	241;sh 269; 331	977	815	[21]	Kaempferol-3-(sinapoyl-diglucoside)-7-glucoside	1285.9 ± 69.8	1203.4 ± 41.9
16 r	23.6	244;sh 268; 330	947	785	[21]	Kaempferol-3-(feruloyl -diglucoside)-7-glucoside	406.5 ± 13.1	406.7 ± 24.1
17 r	23.9	270; 320	917	755	[21]	Kaempferol-3-(p-coumaroyl-diglucoside)-7-glucoside*	122.1 ± 20.1	191.9 ± 24.0
18 r	24.3	266; 328	771	609	[40]	Kaempferol-3-caffeoyldiglucoside*	102.6 ± 16.7	202.2 ± 38.2
19 r	24.6	244;sh 298; 328				Caffeic acid derivative*	385.7 ± 35.2	315.1 ± 28.6
20 r	25.7	256; 328; 354	801	639,		Isorhamnetin-3-*O*-sophoroside-7-*O*-glucoside*	537.2 ± 46.0	113.9 ± 40.5
21 r	26.0	254;sh 268; 352	639	477	[21]	Isorhamnetin-3-*O*-glucoside-7-*O*-glucoside	1257.7 ± 82.9	1243.0 ± 64.2
22 r	28.1	226; 312	581	465, 301, 163		P-coumaric acid	147.2 ± 13.3	165.8 ± 34.9
23 r	29.3	238;sh 298; 328	641	525		Ferulic acid derivative	507.5 ± 26.7	519.4 ± 26.7
			193	178, 149, 134				
24 r	29.6	240;329	701	585		Sinapic acid derivative*	2089.5 ± 114.7	1600.0 ± 50.7
			223	208, 179,164				
25 r	30.1	324				Sinapic acid derivative	241.5 ± 48.5	184.1 ± 19.9
						Hydroxycinnamic acids*	*3708.3 ± 428.9*	*2898.3 ± 149.8*
						Hydroxybenzoic acids*	*105.0 ± 8.9*	*298.0 ± 7.4*
						Flavonols*	*13 726.8 ± 650.4*	*10 755.2 ± 474.3*
						Total polyphenols *	*17 126.4 ± 862.0*	*13 837.6 ± 608.3*

Table 5.3. Continued (* Significant differences (p<0.05) between day 1 and day 10; # [2M−H]−; n.d. not detected).

Peak	Rt	λ max (nn)	$[M - H]^-$	MS/MS	ref.	Tentative identification	Quantification	
							Day 1	Day 10
Wild rocket							***mg.g^{-1} (d.w) ± sd***	***mg.g^{-1} (d.w) ± sd***
1 ^{wr}	14.9	280				Gallic acid derivative*	191.9 ± 2.8	505.9 ± 91.9
2 ^{wr}	18.1	268; 340	787	625,463	[40]	Quercetin-3-*O*- triglucoside	35.2 ± 8.7	31.5 ± 2.5
3 ^{wr}	20.5	266; 330				Non-identified flavonol	35.4 ± 17.7	9.7 ± 3.6
4 ^{wr}	22.5	240; 332				Sinapic acid derivative*	689.7 ± 23.6	251.4 ± 48.5
5 ^{wr}	23.7	328	383	223	[43]	Sinapic acid-*O*-glucoside*	21.4 ± 1.0	7.5 ± 0.1
6 ^{wr}	25.6	266; 338	787	625	[40]	Quercetin-3-*O*- triglucoside	678.6 ± 4.1	717.6 ± 137.1
7 ^{wr}	26.6	272;324				Non-identified flavonol	75.0 ± 1.9	94.4 ± 23.4
8 ^{wr}	29.1	266;332	625	463,301	[40]	Quercetin-3,7-di-*O*-glucoside	192.7 ± 5.3	238.5 ± 47.3
9 ^{wr}	29.9	252; 266; 346				Non-identified flavonol	154.5 ± 10.2	158.9 ± 39.9
10 ^{wr}	30.7	272; 328	993	831,669	[40]	Quercetin-3-sinapoylsophoroside-7-glucoside	3382.9 ± 71.6	3063.2 ± 595.6
11 ^{wr}	31.4	272; 326	963	801,639	[40]	Quercetin-3,4'-diglucoside-3'-(6-feruloy-glucoside)	150.3 ± 10.9	144.9 ± 37.1
12 ^{wr}	32.0	274;324				Quercetin derivative	328.4 ± 4.6	285.3 ± 71.3
13 ^{wr}	32.9	274; 338				Quercetin derivative	108.7 ± 1.7	128.4 ± 38.4
14 ^{wr}	33.5	274; 330				Quercetin derivative	1661.9 ± 28.6	1592.2 ± 305.7
15 ^{wr}	33.9	275; 330				Quercetin derivative	243.9 ± 37.9	289.8 ± 59.8
16 ^{wr}	34.4	274; 330				Quercetin derivative*	86.1 ± 3.4	115.5 ± 18.7
						Hydroxycinnamic acids*	*711.2 ± 24.3*	*257.1 ± 50.5*
						Hydroxybenzoic acids *	*191.9 ± 2.8*	*505.9 ± 91.9*
						Flavonols	*7 169.6 ± 130.6*	*6 902.2 ± 1387.9*
						Total polyphenols	*8 080.9 ± 154.4*	*7 672.3 ± 1529.1*

Table 5.3. Continued (* Significant differences (p<0.05) between day 1 and day 10; # [2M−H]−; n.d. not detected).

Peak	Rt	λ max (nn)	[M - H]$^-$	MS/MS	ref.	Tentative identification	Quantification	
							Day 1	Day 10
Spearmint							***mg.g^{-1} (d.w) ± sd***	***mg.g^{-1} (d.w) ± sd***
1 ^{s}	11.7	280				Catechin derivative	1010.8 ± 128.0	932.7 ± 108.4
2 ^{s}	14.4	244;sh 298; 328				Caffeic acid derivative*	445.5 ± 35.2	356.3 ± 16.4
3 ^{s}	14.7	242;sh 298; 328				Caffeic acid derivative	871.4 ± 60.5	853.9 ± 35.2
4 ^{s}	21.6	236;sh 298; 326	707$^{\#}$	353		Chlorogenic acid*	1709.3 ± 74.8	1553.7 ± 4.5
5 ^{s}	22.0	236;sh 300; 324	345	300,179	[46]	Rosmanol	52.0 ± 8.7	63.6 ± 16.4
6 ^{s}	25.2	342				Caffeic acid derivative*	100.7 ± 5.2	92.5 ±1.3
7 ^{s}	25.9	244;sh 305; 330				Caffeoyl quinic derivative*	41.8 ± 3.4	30.8 ± 2.0
8 ^{s}	26.4	240;sh 300; 326	371		[40]	Hydroxyferuloylglucose*	48.7 ± 4.1	50.1 ± 1.9
9 ^{s}	29.3	284	595	287		Eriodictyol-7-*O*-rutinoside	519.5 ± 43.1	428.7 ± 70.4
10 ^{s}	32.2	255;sh 268; 348	593			Luteolin-7-rutinoside*		
			461	285	[37]	Luteolin-7-glucuronide*	4427.3 ± 249.8	3877.1 ± 114.4
			515			Luteolin-7-glucoside*		
11 ^{s}	33.6	284	609	301		Quercetin-3-O-rutinoside	2565.5 ± 62.7	2641.4 ± 130.3
12 ^{s}	34.6	330;sh 290	359			Rosmarinic acid*	22104.6 ± 1746.1	18691.5 ± 657.8
13 ^{s}	34.9	324;sh 291	537	493,359	[16]	Lithospermic acid*	2069.8 ± 112.0	1780.5 ± 47.0
14 ^{s}	35.3	252;sh 268; 342	607	299,285		Diosmetin-7-*O*-rutinoside	482.6 ± 31.8	464.0 ± 69.3
15 ^{s}	35.6	246;sh 290; 328	633	587, 359		Rosmarinic acid derivative	123.2 ± 12.2	106.4 ± 31.3
16 ^{s}	36.9	315;sh 290				Caffeic acid derivative	72.8 ± 5.4	74.5 ± 2.0
17 ^{s}	37.3	328;sh 290	717	519	[16]	Salvianolic acid	1188.3 ± 690.3	1186.5 ± 257.2
18 ^{s}	37.6	270; 336	461	285	[37]	Kaempferol-3-*O*-glucuronide	520.6 ± 17.3	494.8 ± 80.2
19 ^{s}	38.1	288; 322	495	359	[16]	Rosmarinic acid derivative	3035.2 ± 61.0	3017.9 ± 91.2
20 ^{s}	38.5	326;sh 290	551	359	[16]	Rosmarinic acid derivative	7071.7 ±1345.0	± 5353.6 ± 682.4
21 ^{s}	39.0	290;326	853	359	[16]	Rosmarinic acid derivative*	919.9 ± 175.2	1238.3 ± 54.8
22 ^{s}	39.6	290; 328	568	359	[16]	Rosmarinic acid derivative*	78.7 ± 5.0	98. ± 2.6
						Hydroxycinnamic acids *	*40 572.7 ± 2247.0*	*35 282.5 ± 1316.7*
						Flavone *	*5 429.3 ± 305.9*	*4 661.8 ± 364.1*
						Flavonols	*3086.1 ± 74.5*	*3136.2 ± 185.6*
						Flavan-3-ols	*1 010.8 ± 128.0*	*932.7 ± 108.4*
						Total polyphenols *	*50 099.0 ± 2488.2*	*44 013.2 ± 1926.5*

5.3.2.2 Quantification and stability of phenolic compounds during storage

Once the characterization of phenolic profiles of the PLE extracts of the baby leaf vegetables was accomplished, these compounds were quantified. Calibration curves (7 concentration points from 0.2 to 440 mg mL^{-1}) were constructed for the available commercial standards. The linearity, limits of detection (LOD), limits of quantification (LOQ) and repeatability of the HPLC–DAD method were determined and are shown in Table 5.1, together with their retention time (RT), UV–Vis maxima, $[M-H]^-$ and main fragments ions obtained. R^2 values higher than 0.997 were obtained for all the quantified compounds within the linear range studied. The standards of quercetin and salicylic acid revealed the lowest and the highest LODs and LOQs, respectively. The method showed good repeatability values in the intra-day precision test (0.2-1.1% for retention times and 0.9-2.8% for peak area, n =5), and between 3 consecutive days (inter-day), showing RSD values always below 3.5% (0.2-1.2% for retention times and 0.9-3.5% for peak area, n =15). Tables 5.2 and 5.3 show the results obtained from the quantification of the identified phenolic compounds as well as the total amount of hydroxycinnamic acids, hydroxybenzoic acids, flavones, flavan-3-ols, flavonols and total phenolic compounds determined on each sample. The quantification of the compounds for which there was not a standard available was made using the calibration curve of the compound that was included in their structure. Compounds identified as caffeic acid derivatives (e.g., chicoric acid) were quantified using the caffeic acid calibration curve following the same strategy for the other hydroxycinnamic and hydroxybenzoic derivatives. Flavonols were quantified with the quercetin-3-*O*-glucoside calibration curve in the case of quercetin and isorhamnetin derivatives and with the kaempferol-3-*O*-glucoside calibration curve in the case of kaempferol derivatives. Spinacetin and patuletin were also quantified as a glycosylated flavonol using the quercetin-3-*O*-glucoside calibration curve. On the other hand, flavone compounds were determined using either apigenin or luteolin calibration.

From all the baby leaf vegetables analyzed, the ruby red lettuce had the highest concentration of phenolic compounds (approximately 483 mg g^{-1} (d.w.)), at least 10-times higher than the rest of samples. On the other hand, the lowest phenolic content was found in the wild rocket leaves, where a concentration of 8 mg g^{-1} (d.w.) was found.

Regarding the evolution of the identified compounds during the refrigerated storage, significant changes ($p<0.05$) were found in almost all cases. In green lettuce the total phenolic content increased a 17.5% from 34.1 ± 0.5 mg g^{-1} (d.w.) in the first day of storage to 41.4 ± 9.8 mg.g^{-1} (d.w.) after 10 days, whereas ruby red lettuce phenolic compounds

were shown to be more stable. Nevertheless, there were some significant changes ($p<0.05$) registered in the hydroxycinnamic acids concentration.

Swiss chard, spinach and pea shoot revealed a great stability of their phenolic profile during the 10-day period of refrigerated storage. In fact, their total polyphenols concentration was not significantly modified ($p>0.05$), although some punctual changes were found (marked with an asterisk in Table 5.2). The main components of these samples corresponded to flavonols, that represented a 92.9% and 99% of the total phenolics quantified in spinach and pea shoots, respectively. In spinach PLE extracts, 5,3,4-trihydroxy-3-methoxy-6:7-methylenedioxyflavone-4-glucuronide (17^{sp}) was the major phenolic detected, representing approximately 26% of the total phenolics, which is in agreement with previously published reports [23,24,41].

From all the studied samples, the brassicas had the lowest phenolic content. Within these samples, watercress, mizuna and red mustard had similar contents (21.0 ± 1.9, 18.5 ± 0.3 and 17.1 ± 0.9 $mg.g^{-1}$ (d.w.), respectively). The stability of the phenolic content was different among these samples; while watercress, garden cress and wild rocket total phenolic content was not modified, mizuna and red mustard suffered significant ($p<0.05$) changes during refrigerated storage. In fact, mizuna samples revealed a 7.8% increase, whereas red mustard showed a marked loss (23.7%) in the total phenolic content, as a result of the decrease verified in 4 hydroxycinnamic acids (3^r, 4^r, 19^r and 24^r) and 8 flavonols (5^r, 7^r, 8^r, 10^r, 11^r, 13^r, 17^r and 18^r). In general, flavonols were the main phenolic group in the brassicas leaves studied. More specifically, these compounds represented 65-66% of the phenolics of watercress and mizuna, and 80-90% in red mustard, garden cress and wild rocket. On the other hand, hydroxycinnamic acids, showed a significant evolution during the 10-day period ($p<0.05$), decreasing a 63.8% in wild rocket, a 7.6% in garden cress and 27.9% in red mustard. The opposite behavior was observed for hydroxybenzoic derivatives increasing 54.9%, 72.6%, 64.7% and 62.1% in garden cress, mizuna, red mustard and wild rocket leaves, respectively.

Regarding spearmint, 81% of total phenolic compounds was formed by hydroxycinnamic acids showing a very different composition from all other studied samples, where flavonoids were the main compounds. However, rosmaniric acid (12^s) and the luteolin derivatives (10^s) that represented 44% and 8% of the total phenolic compounds, respectively, decreased during storage. These losses combined with the changes suffered by other minor compounds resulted in a total loss of 13.8% of the phenolic content between day 1 and day 10 of refrigerated storage.

The results obtained for the different samples confirm that the phenolic composition of each vegetable is differently affected during the same storage conditions. The phenolic content of green lettuce, mizuna, red mustard and spearmint changed significantly during storage, while in the other samples only punctual changes in different compounds were noticed. The evolution of the phenolic profile was expected, as they are part of the natural defense metabolism of the plant. Accordingly to previous studies about the stability of phenolic content during storage of green leafy vegetables [13,23,27], several intrinsic factors were pointed out to be determinant to the phenolic compounds metabolism, related to the presence of other compounds with strong antioxidant activity (eg. vitamin C and carotenoids) that could act as a first defense against oxidative stress. In a previous study about the vitamin content of these baby leaf [18], pea shoots, watercress and wild rocket leaves showed the highest content of vitamin C in relation to the other samples, which may have contributed to the stability of their phenolic profile. The fact that hydroxycinnamic and hydroxybenzoic acids undergo more changes, points out to a greater stability of the flavonoids during the refrigerated storage of baby leaves.

5.4 Conclusions

In this work, the use of an optimized PLE method revealed to be an efficient and “green” option to extract polyphenols from green leafy vegetables, being the first time that this method was applied to the phenolic extraction of ready-to-eat baby leaf vegetables. More than 200 polyphenols were tentatively identified in 11 baby leaf vegetables thanks to the application of a HPLC-DAD-MS method. The quantification of these compounds through a 10-day storage period revealed that the evolution of the phenolic profile during refrigerated storage was different in every sample, being flavonols the more stable compounds of the baby leaf samples. In general, the differences between the overall phenolic compositions between the two sampling days were small, although statistically significant for green lettuce, mizuna, red mustard and spearmint, showing that the baby leaf maintained their richness in phenolic compounds that may be beneficial to the human health. Nevertheless, some interesting relationships and variations among the different quantified components have been observed during the studied storage shelf life period.

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CHAPTER 6.

Assessment of nutritional and metabolic profiles of pea shoots: the new ready-to-eat baby leaf vegetable.

This chapter presents the nutritional value and the phytonutrient composition of pea shoots. The stability of the pea shoots quality was studied by comparing levels of different physicochemical traits, nutrients and phytonutrients at the beginning and end of storage.

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Assessment of nutritional and metabolic profiles of pea shoots: the new ready-to-eat baby leaf vegetable.

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Abstract

Pea-shoots are a new option as ready-to-eat baby leaf vegetable. However, data about the nutritional composition and the shelf life stability of these leaves, especially their phytonutrient composition is scarce. In this work, the macronutrient, micronutrient and phytonutrients profile of minimally processed pea shoots were evaluated at the beginning and at the end of a 10-day storage period. Several physicochemical characteristics (colour, pH, total soluble solids, and total titratable acidity) were also monitored. Standard AOAC methods were applied in the nutritional value evaluation, while chromatographic methods with UV-Vis and mass detection were used to analyse free forms of vitamins (HPLC-DAD-ESI-MS/MS), carotenoids (HPLC-DAD-APCI-MS^n) and flavonoid compounds (HPLC-DAD-ESI-MS^n). Atomic absorption spectrometry (HR-CS-AAS) was employed to characterize the mineral content of the leaves. As expected, pea leaves had a high water (91.5%) and low fat (0.3%) and carbohydrate (1.9%) contents, being a good source of dietary fibre (2.1%). Pea shoots showed a high content of vitamins C, E and A, potassium and phosphorous compared to other ready-to-eat green leafy vegetables. The carotenoid profile revealed a high content of β-carotene and lutein, typical from green leafy vegetables. The leaves had a mean flavonoid content of 329 mg/100 g of fresh product, mainly composed by glycosylated quercetin derivatives. Pea shoots kept their fresh appearance during the storage being colour maintained throughout the shelf life. The nutritional composition was in general stable during storage, showing some significant ($p<0.05$) variation in certain water-soluble vitamins.

Keywords: Pea shoots; nutritional composition; vitamins and minerals; carotenoid profile; flavonoid profile; storage.

6.1 Introduction

The consumption of green leafy vegetables is recommended due to their high content of vitamins, minerals and antioxidant phytochemicals, as well as low content of fat and carbohydrates (Rico, Martín-Diana, Barat, & Barry-Ryan, 2007). Minimally processed vegetables sold as ready-to-eat salads are a convenient way to include vegetables in the diet. To increase variety and attract even more consumers, the fresh-cut producers seek for new varieties of leafy vegetables to add to ready-to-eat salad mixtures (Martínez-Sánchez et al., 2012). Pea shoots were recently presented as a ready-to-eat vegetable, and are recognized as a popular specialty vegetable in some parts of Asia and Africa that is gaining popularity in the United States and Europe (Miles & Sonde, 2003).

Peas (*Pisum sativum*) are among the most consumed vegetables worldwide, with a registered global production of 15 million tonnes in 2010 (FAO, 2013). It is normally consumed as a seed food, and is a good source of proteins, vitamins and minerals (Martins, 2010). The consumption of leaves of the pea plants, also known as pea shoots, is not as common as eating the peas. They are harvested in a very early maturation stage, when the leaves and tendrils are tender, crispy and have an intense pea flavour (Miles & Sonde, 2003). This baby leaf green leafy vegetable can be eaten raw in salads, or cooked with others ingredients ("Pea shoots," 2013). Accordingly to Miles and Sonde (2003), pea shoots are a very perishable product with a high market value, when compared to other common leafy vegetables. As a minimally processed vegetable, pea leaves can be packed solely or in ready-to-eat salad mixtures and their quality and safety is strictly dependent on the maintenance of refrigerating conditions during storage (Rico et al., 2007).

The pea plant is one of the most-studied vegetables, being a well-established classic model for genetics and agronomic studies (Edelenbos, Christensen, & Grevsen, 2001; Hamada & El-Enany, 1994; Wong, Bhalla, Ottenhof, & Singh, 2008). Its origins are in Middle East and Mediterranean regions, integrating the diet of early civilizations (Smýkal, Coyne, Redden, & Maxted, 2013). The nutritional composition of peas is published in official nutritional tables (Martins, 2010). On the other hand, the nutritional quality of pea shoots is not mentioned. There are however some nutritional allegations of being rich in vitamin C and A in the producers' *website* ("Pea shoots," 2013). Specific scientific data regarding the nutritional composition of pea shoots is scarce, being most of the available information based in the generalization of the green leafy vegetables composition (Miles & Sonde, 2003). In this context, the objective of this work was to characterize and compare physicochemical characteristics as well as nutritional quality

and phytonutrients composition of minimally processed pea shoots stored under refrigerated conditions. Colour, total soluble solids (TSS), total titratable acidity (TTA), pH, macronutrient composition and also minerals, vitamins, carotenoids and flavonoids contents of pea shoots were assessed.

6.2 Material and Methods

6.2.1 Samples

Minimally processed pea shoots (*Pisum sativum*) were obtained from a producer (Odemira, Portugal). Upon arrival to the laboratory, one day after processed (washed, cut and packed), pea shoots were divided in two groups. One was prepared for analysis and the second was stored under refrigerated conditions (3 ± 1 ºC) for 10 days. About 200g of fresh leaves from each group were used for colour, TTS, TA, pH and macronutrient analyses. Vitamins, minerals, carotenoids and flavonoids were determined in freeze-dried pea shoots samples (Telstar Cryodos-80, Terrassa, Barcelona), that were powdered in a knife mill (GM 200, RETSCH, Haan, Germany) and stored protected from light, oxygen and heat until analysis.

6.2.2 Quality analysis

6.2.2.1 Physicochemical characteristics

Leaves colour parameters L*, a* and b* were determined with a tristimulus colorimeter (CR-400Chroma Meter, Konica Minolta, Japan), where L* defines the lightness (0<L*<100) variation. Parameters a* define the red (+) to green (-) and b* the blue (-) to yellow (+) chromaticity. These were used to calculate the hue angle (h^o= arctang (b*/a*)) and chroma (C*= (a*2 + b*2)1/2) values. The equipment was set up for illuminant D65 with 10º observer angle and calibrated using a standard white plate. Forty measurements were made in different leaves at each sampling day. Total soluble solids (TSS) were determined on pea shoots juice with a Digital Refractometer (ºBrix, HI 9680, Hanna Instruments, USA). The pH was measured with a pH-meter (Crison Instruments, Barcelona, Spain) in 10 g of leaves homogenized in 20 mL of deionised water (AOAC, 2000). Total titratable acidity (TTA) was determined by titration with 0.1 M NaOH to pH 8.1 (AOAC, 2000) and expressed as the units of citric acid (mg/100g) on a fresh weight (f.w.) basis.

6.2.2.2 Nutritional Composition

The water, protein (factor of 6.25), fat, ashes and total dietary fibre contents were determined accordingly to the AOAC (2000) methods, in the samples after one and ten days of storage. Protein content was estimated by the Kjeldahl method, fat by Soxhlet extracting method, whereas ash content was determined by incineration at 600±15 °C and dietary fibre by an enzymatic gravimetric method. All values were presented as a percentage, being carbohydrates calculated by difference. All proximate composition analyses were done, at least, in triplicate. Energy was calculated according Atwater Factors (Otten, Hellwig, & Meyers, 2006).

Mineral composition was evaluated by a High Resolution-Continuum Source Atomic Absorption Spectrophotometric (HR-CS-AAS) method optimized by Santos, Oliva-Teles, Delerue-Matos, and Oliveira (2014). Briefly, 150 mg of freeze dried pea shoots were digested with 9 ml of nitric acid diluted with ultrapure water (43.3%) by microwave assisted digestion (MARS-X, CEM, Mathews, NC, USA). Potassium, sodium, calcium, magnesium, iron, manganese and zinc were analysed with flame atomization (FAAS) (ContrAA 700, Analytik Jena, Germany), while copper was determined by electrothermal (EAAS) atomization. Phosphorous content was measured according to the 4500-P standard method (Greenberg, Clesceri, & Eaton, 1992) the vanadomolybdophosphoric acid colorimetric method in a UV-Vis spectrophotometer (Evolution™ 300, Thermo Scientific, Waltham, MA, USA). Four replicates of pea shoots from each sampling day were used in minerals determination.

Several free forms of water-soluble vitamins (C, B_1, B_2, B_3, B_5, B_6 and B_9) and fat-soluble vitamins (Pro-vitamin A and E (α-tocopherol)) were assessed by HPLC-MS/MS and HPLC-DAD methods described by Santos, Mendiola, Oliveira, Ibáñez, and Herrero (2012). Briefly, 250 mg of freeze dried sample was extracted with 16 mL of 10 mM ammonium acetate/methanol 1:1 (v/v) in an ultrasound bath for 15 minutes. After centrifugation (14000 g; 15 min) the supernatant was concentrated under nitrogen stream and injected into a HPLC–ESI-MS/MS system (Thermo Scientific, San Jose, CA, USA) to determine the water-soluble vitamin content. The solid residue was re-extracted twice with ethyl acetate (0.1% BHT) (6 + 6 mL) in an ultrasound bath (15 min). After centrifuged (14000g, 15 min.), the two supernatants were combined and dried under nitrogen stream. The residue was re-dissolved in 3 mL of ethyl acetate and injected in a HPLC-DAD system (Agilent 1100 Santa Clara, CA, USA) to determine fat-soluble vitamin content of the samples. Pea shoot vitamin contents were determined along the storage period (day 1 and day 10). The results were expressed as mg /100 g, with exception for vitamin A,

expressed as mg Retinol Activity Equivalent (RAE) calculated accordingly to the following equation: 1 mg RAE = 12 mg β-carotene (Otten et al., 2006).

6.2.2.3 Carotenoid profile

The extraction procedure used to study the carotenoid profile was described previously for the analysis of fat-soluble vitamins (Santos et al., 2012). Once re-dissolved, the extract was filtered through a 0.45 µm nylon filter and injected in a HPLC-DAD-APCI-MS^n system. The equipment used was an Agilent 1200 liquid chromatograph (Agilent, Santa Clara, CA, USA) equipped with an autosampler, a DAD, and directly coupled to an ion trap mass spectrometer (Agilent ion trap 6320) via an atmospheric pressure chemical ionization (APCI) interface, using an YMC C30 analytical column (5 µm particle size, 250 x 4.6 mm i.d.) (YMC, Schermbeck, Germany). The mobile phases (A: MeOH/water, 90:10 v/v; B: MTBE/MeOH/water, 90:6:4, v/v/v) eluted in the following gradient: 0 min, 6.5 %B; 8 min, 6.5 %B; 43 min, 100 %B; 46 min, 6.5 %B; 55 min, 6.5 %B. The flow rate was 1 mL min^{-1} and the injection volume 10 µL. The diode array detector recorded the spectra from 220 to 700 nm, and the chromatograms were monitored at 450 nm. MS analysis was conducted with APCI in positive ionization mode using the following parameters: capillary voltage, −3.5 kV; drying temperature, 350 °C; vaporizer temperature, 400 °C; drying gas flow rate, 5 L/ min; corona current (which sets the discharge amperage for the APCI source), 4000 nA; nebulizer gas pressure, 60 psi. A range from m/z 150 to m/z 1300 was acquired and MS/MS automatic mode was used on the more abundant ions in the MS spectra to identify the principal fragmentation ions. The major carotenoids were identified by combining absorption spectroscopic data, chromatographic properties and mass spectra (MS) information with the values obtained from available standards and data reported in the literature. To quantify the carotenoids, six different concentrations were used to construct a calibration curve of lutein (linear range 10-200 µg mL^{-1}, R^2>0.998) and β-carotene (6.25 – 250 µg mL^{-1}, R^2>0.999). All xanthophylls were quantified as lutein equivalents, while the carotene isomers were quantified as β-carotene equivalents. The results were expressed in mg/ 100 g of fresh weight (f.w.), as mean ± standard deviation of two extracts from each sampling day.

6.2.2.4 Flavonoid compounds characterization

The flavonoids present in pea shoots were analysed by an HPLC-DAD-ESI-MS^n method. Briefly, 500 mg of freeze dried pea shoots were extracted with 70% MeOH in a pressurized liquid extraction system (ASE 200, Dionex, Sunnyvale, CA, USA) with 11 mL extraction cells and following a procedure previously described (Miron, Plaza, Bahrim,

Ibáñez, & Herrero, 2011). The extraction conditions were the following: extraction time, 20 min; temperature, 70ºC; pressure 10 MPa; flush volume, 60%. The extracts were first dried in a Rotavapor R-210 (Büchi, Labortechnik AG, Flawil, Switzerland) and later freeze-dried (Labconco Corporation, Missouri, USA). The dried extracts were re-dissolved in 70% MeOH (5 mg mL^{-1}) and filtered through a 0.45 µm disposable syringe filter. For the study of acyl flavonoid derivatives, an alkaline hydrolysis was carried out to eliminate acid moieties (p-coumaroyl, caffeoyl, feruloyl and sinapoyl), following the procedure described by Francisco et al. (2009).

The analyses of both extracts (native and hydrolyzed) were carried out on an Agilent 1200 liquid chromatograph (Agilent, Santa Clara, CA, USA) equipped with an autosampler, a DAD, and directly coupled to an ion trap mass spectrometer (Agilent ion trap 6320) via an electrospray interface. The column was a Zorbax Eclipse XBD C18 (5 µm, 150×4.6 mm) (Agilent, Santa Clara, CA) and the mobile phases (A: 0.1% formic acid; B: MeOH with 0.1% formic acid), eluted with the following gradient: 0 min, 95% A; 4 min, 95% A; 20 min, 73% A; 50 min, 5% A; 57 min, 99% A; 58 min, 99% A; 60 min, 95% A. A flow rate used was of 0.7 mL min^{-1} and the injection volume of 10 µL. The UV-Vis spectra were recorded from 200 to 550 nm and the chromatograms were monitored at 330 nm. The MS detector operated under ESI negative ionization mode, with dry temperature of 350 ºC; dry gas flow of 12 L min^{-1}; nebulizer gas pressure of 40 psi and 3500 V of capillary voltage. A mass scan range was set from m/z 100 to 1000 and MS/MS automatic mode was also used. The flavonoids were characterised according to their retention time, UV-Vis and mass spectra compared to information available in the literature and available commercial standards. To quantify the flavonoid contents, a calibration curve was obtained from seven different concentrations of quercetin-3-O-glucoside (linear range: 1.0-66.7 µg mL^{-1}, R^2>0.999) and kaempferol-3-O-glucoside (linear range: 0.2-14.8 µg mL^{-1},R^2>0.999). The results were expressed in mg/100 g of fresh weight (f.w.), as mean ± standard deviation of two extracts.

6.2.3 Statistical Analysis

Data were expressed as mean ± standard deviation and the differences between the two days of sampling (day 1 and day 10) were tested by the one-way ANOVA. Normal distribution of data in the different samples was assessed by Kolmogorov–Smnirnov test. Statistical significance was defined for a $p<0.05$ (95% confidence level). The statistical analyses were carried out using the Statistica 8.0 software (Statsoft Inc., Tulsa, USA).

6.3 Results and Discussion

Convenience is the key factor that leads consumers to choose minimally processed vegetables, increasing the intake of fresh products in their diets (Barrett, Beaulieu, & Shewfelt, 2010). Although it could not be directly appreciated by the consumer, the nutritional quality of these products is also becoming a choosing factor, due to an increased perception of the possibility of preserving health by choosing a balanced diet (Poiroux-Gonord et al., 2010). Due to the lack of specific information about the nutritional composition of pea shoots, this work focused on achieving a comprehensive characterization of nutritional quality of these leaves. Micronutrient composition (vitamins and minerals) of pea leaves was evaluated, knowing that it is determined by genetic factors, but it also reflects the agronomic practices and environmental conditions during their growth and storage (Hanson, Yang, Chang, Ledesma, & Ledesma, 2011; Santos et al., 2012). Carotenoids and flavonoids are phytonutrients with recognized beneficial antioxidant properties (Poiroux-Gonord et al., 2010). Their content is also affected by intrinsic and external factors. The levels of each nutrient were compared during the storage period to evaluate the stability of the bioactive compounds.

6.3.1 Evolution of physicochemical characteristics

The evolution of physicochemical characteristics analysed in pea shoot is presented in Table 6.1. These parameters monitored the evolution of quality characteristics with major influence on the consumer's choice: colour/appearance and flavour (Barrett et al., 2010). The colour of minimally processed vegetables can suffer changes during storage, being the loss of greenness and the appearance of a yellowish tonality signs of the onset of senescence reactions (Barrett et al., 2010; Kidmose, Edelenbos, & R., 2002). Pea shoots leaves showed a dark green colour (Hue angle of 122º) that was constant during the storage period. No significant differences ($p<0.05$) were found between a* values from the beginning and end of storage, which corroborates the preservation of the green tonality. Relatively to the other colour parameters, the changes were less than 6% of the initial value recorded, being the fresh appearance maintained throughout the refrigerated storage. The levels of TSS revealed a slight decrease (-18%) during storage, being followed by a 20% increase of the TTA (Table 6.1). The variation of the pH was less pronounced (-4%), which could also point to an overall preservation of flavour characteristics, when leaves are stored under low temperatures (3±1ºC). These results highlight the possibility of preserving the pea shoots quality for at least 10 days after harvested, when properly processed and stored.

Table 6.1 Variation of physicochemical parameters through the storage period (mean value ± standard deviation; * means no significant variation between sampling days ($p<0.05$); TSS: total soluble solids; TTA: total titratable acidity).

	Pea shoots	
	day 1	*day 10*
Colour parameters		
L	45.9±2.3	47.3±2.0
a	-18.6±0.9*	-18.8±0.6*
b	29.4±2.2	31.4±2.2
C	34.8±2.1	36.6±2.1
Hue angle	122.3±1.7	120.9±1.4
TSS (%)	6.28±0.15	5.34±0.26
pH	6.42±0.07	6.18±0.05
TTA(%)[1]	0.18±0.03	0.22±0.01

[1] % of citric acid

6.3.2 Nutritional Composition

The macro and the micronutrient contents of pea shoots are presented in Table 6.2. The macronutrient composition was similar to other baby leaf vegetables, like spinach, lamb's lettuce or watercress (Martins, 2010). However, these leaves showed a higher percentage of protein (approximately 3%) and dietary fibre (approximately 2%) than the ones found in lettuces (1.8% and 1.3% of protein and dietary fibre, respectively), commonly present in ready-to-eat salads (Martins, 2010). All macronutrients showed a stable content during storage.

Regarding the micronutrient composition, pea shoots showed to be a good source of vitamin C, Vitamin E and vitamin A. Vitamin C represented more than 96% of the total vitamin content, followed by vitamin E and vitamin A. These vitamins levels were higher than the ones found in other common green leafy vegetables, especially in the case of vitamin C (Santos et al., 2012). Regarding the water-soluble vitamins from the B group, the highest values were found for pantothenic acid (B_5). The mineral composition revealed that pea shoots are also a good source of potassium and phosphorous. On the other hand, sodium, calcium and magnesium contents were lower than the levels found in other baby leaf vegetables (Santos et al., 2014). Concerning the microminerals (iron, manganese, zinc and copper), they represent 0.5% of the total mineral content determined. Iron was the most abundant, but zinc levels were higher than those reported in other common baby leaf vegetables (Santos et al., 2014), revealing that these leaves

could enhance the zinc content of a ready-to-eat salad. In this sense, the micronutrient composition, especially vitamin C, vitamin E, vitamin A, potassium and zinc contents, are the most distinguishable characteristics of pea shoots in relation to other baby leaf vegetables.

Table 6.2 Nutritional composition of pea shoots during storage (mean value ± standard deviation relative to fresh weight (f.w.); * means a significant variation ($p<0.05$) between sampling days).

	Pea shoots	
	day 1	day 10
Macronutrient composition	**%**	
Water	91.5 ± 0.2	91.6 ± 0.1
Protein	4.0 ± 1.0	3.1 ± 0.2
Fat	0.3 ± 0.0	0.4 ± 0.0
Ash	0.9 ± 0.0	0.7 ± 0.1
Dietary Fibre	2.0 ± 0.1	2.2 ± 0.1
Sugar (by difference)	1.2 ± 0.9	2.0 ± 0.2
Energy[1] (/100g f.w)	*24.1 KCal (102.2kJ)*	
Micronutrient composition	**mg/100g f.w.± sd**	
Water-soluble vitamins		
Ascorbic Acid (C)	153.94 ± 2.81	174.05 ± 19.30
Thiamine (B_1)	0.18 ± 0.02	0.19 ± 0.01
Riboflavin (B_2)	0.13 ± 0.04	0.13 ± 0.01
Nicotinamide (B_3)	0.14 ± 0.02*	0.10 ± 0.00*
Pantothenic Acid (B_5)	0.64 ± 0.03*	1.19 ± 0.03*
Pyridoxine (B_6)	0.02 ± 0.00*	0.05 ± 0.00*
Fat-soluble vitamins		
Vitamin E (α-tocopherol)	2.65 ± 0.09*	3.66 ± 0.07*
Vitamin A (RAE eq.)[2]	1.42 ± 0.01	1.36 ± 0.06
Minerals		
Potassium	315.97 ± 8.30*	332.4 ± 6.02*
Sodium	5.08 ± 0.24*	6.46 ± 0.22*
Calcium	57.59 ± 2.69	58.17 ± 2.92
Magnesium	27.20 ± 1.60	27.84 ± 0.92
Phosphorus	96.14 ± 1.81	90.12 ± 7.61
Iron	1.47 ± 0.02	1.48 ± 0.02
Manganese	0.35 ± 0.02	0.33 ± 0.01
Zinc	0.46 ± 0.03	0.49 ± 0.01
Copper	0.14 ± 0.01*	0.12 ± 0.00*

[1] Calculated accordingly to Atwater Factors (Otten et al 2006).
[2] RAE = Retinol activity equivalent

The nutritional quality of pea shoots revealed some variations throughout storage, mostly in their vitamin content. Pyridoxine revealed the highest variation (+57%) between sampling days, followed by pantothenic acid (+46%), nicotinamide (-35%) and vitamin E (+28%). This behaviour was also described in other leafy vegetables, being referred in some studies the occurrence of vitamin synthesis in the post-harvest period (Sánchez-Mata, Cámara, & Díez-Marqués, 2003; Santos et al., 2012). Moreover the enzymatic action against the conjugated form of the vitamins could originate higher levels of free form of the vitamin throughout the storage (Hounsome, Hounsome, Tomos, & Edwards-Jones, 2009; Santos et al., 2012). The mineral content showed less variation, and could be considered stable during the studied period.

6.3.3 Carotenoid profile

The fat-soluble pigments extracted from pea shoots (carotenoids and chlorophylls) were successfully separated by the chromatographic conditions employed (see Figure 6.1). From the obtained UV-Vis spectra information it was possible to identify 26 compounds, corresponding 12 to carotenoids and the other to chlorophyll a and b and their derivatives.

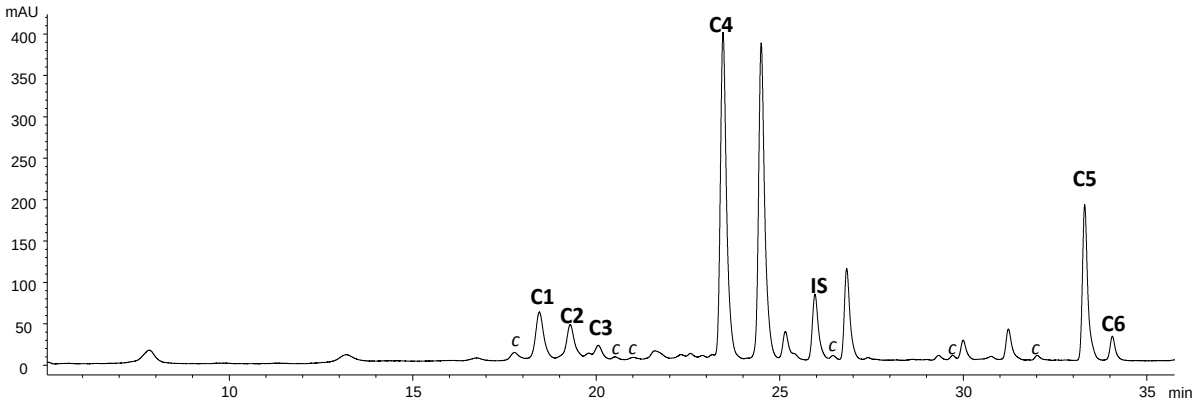

Figure 6.1 HPLD-DAD chromatogram (450 nm) of the carotenoid profile of peas shoots. For peak identification and information see Table 6.3 (*c*, correspond to carotenoid compounds not completely identified).

A tentative identification of the six main separated carotenoid compounds was accomplished by comparing the UV-Vis and MS spectra information provided by the two detectors (i.e., DAD and MS) with commercial standards and data from the literature (Britton, Liaaen-Jensen, & Pfander, 2004; Castro-Puyana et al., 2013; Crupi, Milella, & Antonacci, 2010). Information about characteristic UV–Vis spectra, $[M+H]^+$, and the main fragments obtained by MS^2 experiments for the different carotenoids is presented in Table

6.3, together with their quantification in the two sampling days. In MS detection the carotenoids were detected as protonated molecules $[M+H]^+$, with exception of lutein (peak C4) identified by the dehydrated fragment ion $[M+H-H_2O]^+$ (Castro-Puyana et al., 2013). This ion was also detected as a fragment ion in the other hydroxylated carotenoids (peaks C1, C2 and C3) at a m/z of 583 $[M+H-H_2O]^+$. These three compounds had the same protonated molecules at a m/z of 601 $[M+H]^+$ and share the same fragmentation ions. Their identification was based on the different relative intensities of the main MS^2 fragment ions and on their maxima UV-Vis spectra. The fragmentation profile of violaxanthin (C1) showed five different main ions (Table 6.3), while in the other xanthophylls (neoxanthin (C2) and luteoxanthin (C3)) only 3 of those ions were present. The compounds C5 and C6 also presented a similar MS spectrum, however, in this case the presence in compound C6 of shift of absorption maxima (approximately 4 nm with respect to the C5) and a low *cis* peak at 342 nm permitted the identification of *cis*-β-carotene.

Table 6.3 Carotenoids from pea shoots: retention time (Rt), UV-Vis maxima, mass spectral data, tentative identification and concentration (mean value ± standard deviation relative to fresh weight (f.w.); * means a significant variation ($p<0.05$) between sampling days).

Peak	**Rt**	**λ max** (nn)	**$[M+H]^+$** (m/z)	**MS^2** main fragment ions (m/z)	**Identification**	**Quantification**	
						Day 1	Day 10
						mg/100 g	***mg/100 g***
C1	18.4	416, 440, 469	601.3	583.4, 565.3 509.4, 491.3, 221.0	Violaxanthin	0.3 ± 0.1	0.6 ± 0.3
C2	19.3	414, 436, 464	601.5	583.4, 565.4 491.2	Neoxanthin	1.5 ± 0.1	1.6 ± 0.0
C3	20.0	398, 422, 449	601.3	583.4, 491.4, 221.1	Luteoxanthin	0.4 ± 0.2*	1.2 ± 0.3*
C4	23.5	423sh, 445, 473	551.4[a]	551.4, 533.4, 495.3,429.3	Lutein[b]	12.1 ± 0.2	12.7 ± 0.5
C5	33.3	428sh, 452, 479	537.4	537.4, 481.2, 441.4, 399.2	all-*trans*-β-Carotene[b]	13.9 ± 0.1	13.2 ± 0.6
C6	34.1	342, 424sh, 448, 472	537.4	537.4, 481.3, 444.3, 413.3	*cis*-β-Carotene	3.1 ± 0.0	3.1 ± 0.1
					Total Carotenoides	*31.3 ± 0.6*	*32.4 ± 0.6*

[a] $[M+H-H_2O]^+$
[b] Identification corroborated using commercial standards
sh, spectral shoulder

Pea shoots revealed a mean carotenoid content of 31.9±0.8 mg/100g (f.w.), being β-carotene and lutein the main carotenoids of the pea leaves composition, representing each about 40% of the total carotenoid content (Table 6.3). The other xanthophylls were present in lower amounts, being this a typical carotenoid profile of a dark green leafy

vegetable (Reif, Arrigoni, Schärer, Nyström, & Hurrell, 2013). Although there is no specific value proposed for the daily intake of carotenoids, there are epidemiological evidence suggesting that higher blood concentrations of β-carotene and other carotenoids obtained from foods are associated with a lower risk of several chronic diseases and reduction of eye diseases (Otten et al., 2006). In this sense, pea shoots can be considered a good source of these compounds, especially β-carotene, that has provitamin-A activity, and lutein that is a component of the human retina. Besides, all carotenoids would contribute to the antioxidant properties of this product.

The carotenoid degradation is common during the senescence of the leaves, being also affected by the presence of light during post-harvest storage (Kidmose et al., 2002). In pea shoots, the global carotenoid content was stable during the storage period, revealing, once more, the stability of the nutritional quality of this product.

6.3.4 Flavonoid characterization

The presence of several flavonoid glycosides was observed in the native extract of pea shoots. However, before the characterization of the compounds in the native extract, the deacylated compounds were studied in the hydrolysed extract. In this extract 5 major compounds were identified (see Figure 6.2), according to their UV-Vis spectra as flavonol-3-O-glycosides (peaks 3 and 5) and hydroxycinnamic acids (peaks 1, 2 and 4) (Table 6.4).

The MS analysis of the flavonols revealed the presence of a molecular ion at m/z 787 ($[M-H]^-$) and m/z 771 ($[M-H]^-$) for peak 3 and 5, respectively. The ions obtained in the MS^2 experiment allowed the identification of these compounds as quercetin (peak 3) and kaempferol (peak 5) glycosylated with a sophorotrioside unit (Table 6.4). The fragmentation pattern obtained was in agreement with the results described in the literature for the identified compounds (Ferreres, Llorach, & Gil-Izquierdo, 2004). The other compounds found in the hydrolyzed extract correspond to p-coumaric acid (m/z 163, $[M-H]^-$), ferulic acid (m/z 193, $[M-H]^-$) and sinapic acid (m/z 223, $[M-H]^-$) (Figure 6.2).

The analysis of flavonoid compounds present in the native extract permitted to detect, by their typical UV spectra, 5 flavonol 3-O-glycosides compounds (peaks 3, 5, 6, 10 and 14) and 7 peaks of acylated flavonols (compounds 7, 8, 9, 11, 12, 13 and 15). The first group presented an UV-Vis spectra with a maximum between 350 and 385 nm, while the second group had a UV-Vis spectral shape that resembles the overlapping of a flavonol spectrum with a hydroxycinnamic acid, with a maximum around 310–330 nm (Carazzone, Mascherpa, Gazzani, & Papetti, 2013).

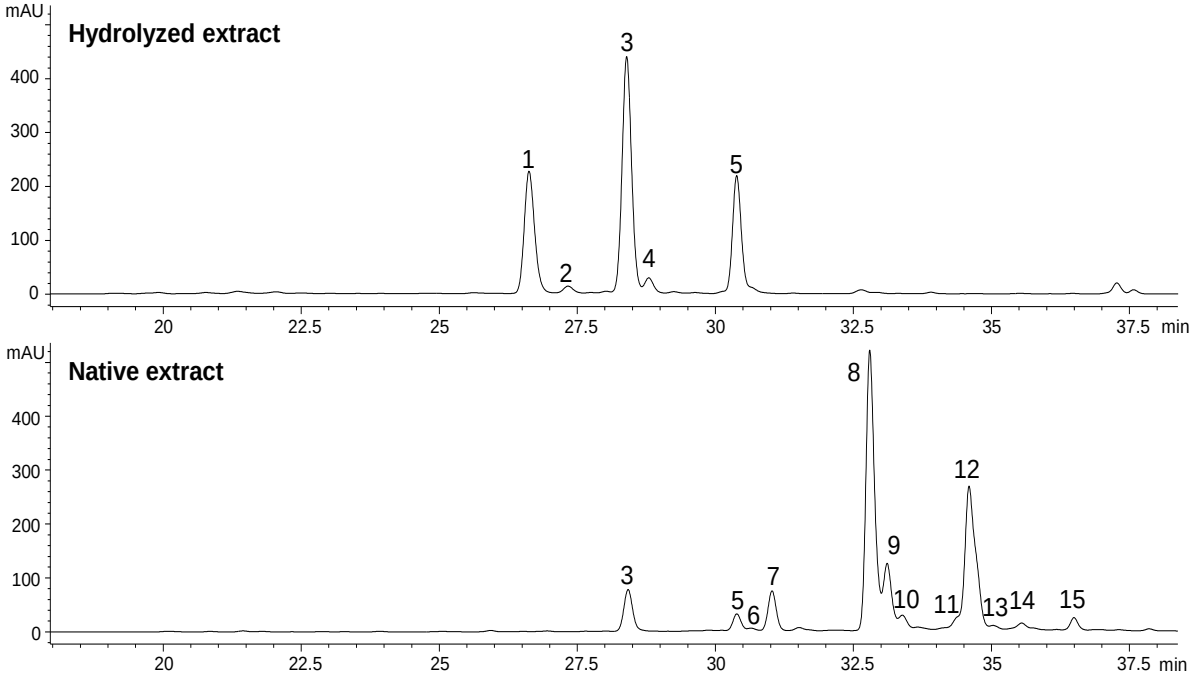

Figure 6.2 HPLD-DAD chromatograms (330 nm) of the hydrolysed and native extracts of pea shoots. *Compounds identification:* 1- p-coumaric acid; 2- ferulic acid; 3- quercetin-3-O-sophorotrioside; 4- sinapic acid; 5- kaempferol-3-O-sophorotrioside; 6- isomer from 5; 7- quercetin-3-(caffeoyl-diglucoside)-7-glucoside; 8- quercetin-3-(p-coumaroyl-diglucoside)-7-glucoside; 9- quercetin- 3-feruloylsophoroside-7-glucoside; 10- quercetin-3-O-(glucuronide-diglucoside)-7-glucoside; 11- kaempferol-3-sinapoylsophotrioside; 12- kaempferol-3-(p-coumaroyl-diglucoside)-7-glucoside; 13- kaempferol-3-feruloylsophoroside-7-glucoside; 14- kaempferol-3-O-sophorotrioside-7-glucoside; 15– isomer from 12.

The MS analysis allowed to identify these compounds as quercetin and kaempferol derivatives. Compounds 3 and 5, previously identified in the hydrolysed extract, were also present in the native pea shoots extract but in minor amounts. The acylated flavonols were the main compounds in the flavonoid profile of pea shoots (see Figure 6.2), especially the compound 8 (m/z 933 $[M-H]^-$) identified as quercetin-3-(p-coumaroyl-diglucoside)-7-glucoside and compound 12 (m/z 917 $[M-H]^-$) identified as kaempferol-3-(p-coumaroyl-diglucoside)-7-glucoside. In both compounds the MS^2 experiment revealed a loss of 146 mu, corresponding to the loss of a p-coumaroyl unit (Table 6.4). The presence of these compounds was also described in the flavonoid composition of other green leafy vegetables (Lin, Sun, Chen, & Harnly, 2011).

Table 6.4 Flavonoids from pea shoots: retention time (Rt), UV-Vis maxima, mass spectral data, MS2 fragmentation pattern and tentative identification. Peak numbers as in Figure 6.2 (sh, spectral shoulder).

Peak	Compounds	RT (min.)	UV–Vis maxima (nm)	$[M-H]^-$ (m/z)	MS^2 main fragment ions (m/z) (%)				
	Glycosylated flavonoid from pea shoots hydrolyzed extract								
					(-120)	**(-162)**	**(-176)**	**(-342)**	**(-486)**
3	Quercetin-3-O-sophorotrioside	28.8	256; sh 268; 352	787	667 (100)	625 (100)		445 (30)	301 (19)
5	Kaempferol-3-O-sophorotrioside	30.7	266; 347	771	651 (23)	609 (100)		429 (22)	285 (22)
	Others Glycosylated flavonoid from pea shoots native extract								
10	Quercetin-3-O-(glucuronide-diglucoside)-7-glucoside	33.6	256; sh268; 354	963			787(100)		
14	Kaempferol-3-O-sophorotrioside-7-glucoside	35.8	266; 349	933		771 (100)			
	Acylated Flavonoid derivatives from pea shoots native extract								
					(-146) (-p-coumaroyl)	**(-162)** (-caffeoyl)	**(-176)** (-feruloyl)	**(-206)** (-sinapoyl)	**(-338)** (-feruloyl-glucoside)
7	Quercetin-3-(caffeoyl-diglucoside)-7-glucoside	31.3	252; sh 268;332	949		787 (100)			
8	Quercetin-3-(p-coumaroyl-diglucoside)-7-glucoside	33.1	226; 316	933	787 (100)				
9	Quercetin- 3-feruloylsophoroside-7-glucoside	33.3	251; sh 268; 331	963			787 (100)		
11	Kaempferol-3-sinapoylsophotrioside	34.1	266; 336	979				771 (100)	
12	Kaempferol-3-(p-coumaroyl-diglucoside)-7-glucoside	34.9	268; 316	917	771 (100)				
13	Kaempferol-3-feruloylsophoroside-7-glucoside	35.3	268; 320	947			771 (100)		609(5)

Pea shoots had a mean flavonoid content of 329 ± 1 mg/100 g (f.w) (see Figure 6.3), which indicates pea shoots as a good source of these antioxidant compounds. Quercetin derivatives represent 67% of the total flavonoids. During the storage, no significant changes ($p<0.05$) were observed, in agreement with other reports that mention a greater stability of the glycosylated flavonoids in relation to other phenolic compounds like the hydroxybenzoic or hydroxicinnamic acids (Martínez-Sánchez, Marín, Llorach, Ferreres, & Gil, 2006).

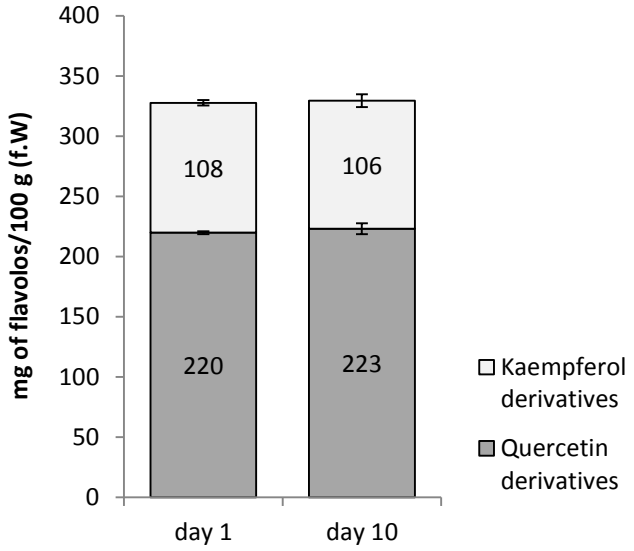

Figure 6.3. Flavonoid content from pea shoots at the beginning and at end of storage period.

6.4 Conclusions

The results achieved in the present study demonstrated that fresh pea shoots are a good source of different micronutrients, with significant amounts of biologically active compounds. The inclusion of these leaves in the diet can contribute to a higher intake of antioxidant compounds like flavonoids, carotenoids and vitamin C, and to the daily requirements of minerals, especially potassium. As a minimally processed vegetable, the pea shoots showed a very good stability of their main quality characteristics, when stored under refrigerated conditions for 10 days. The results presented in this work can be also useful to complete food composition databases with the inclusion of a new option of a nutritious green leafy vegetal.

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CHAPTER 7. Ready-to-eat salads: Adding fresh-cut aromatic herbs improves their nutritional value?

This chapter presents the nutritional quality evolution of 4 fresh-cut aromatic herbs during storage. Several physicochemical traits, nutrients, phytonutrients and antioxidant capacity were assessed to investigate the nutritional benefits of adding aromatic herbs to ready-to-eat salads.

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Ready-to-eat salads: Adding fresh-cut aromatic herbs improves their nutritional value?

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Abstract

Ready-to-eat salads are a successful convenient healthy food. Producers and consumers are always looking for more attractive mixtures with improved flavour and quality. In this work, the objective was to evaluate the nutritional quality of aromatic herbs added to ready-to-eat salads, and their stability during shelf life. Several physicochemical quality characteristics (colour, pH, total soluble solids, and total titratable acidity) were monitored in four fresh-cut aromatic herbs (chives, coriander, spearmint and parsley) during 10 days under refrigerated conditions. The nutritional composition of the samples was determined, including their mineral composition (phosphorous, potassium, sodium, calcium, magnesium, iron, zinc, manganese and copper) and fat- and water-soluble vitamins. The total soluble phenolics, flavonoids and the antioxidant capacity were also determined by spectrophotometric methods. The aromatic herbs kept their fresh appearance during the storage period, being their colour maintained throughout the shelf life. Moisture, protein, fat, dietary fibre and mineral content were stable during storage. Coriander leaves possessed the highest content of minerals and fat-soluble vitamins, while spearmint showed the best scores in the phenolic and flavonoid contents as well as in the antioxidant capacity assays. Vitamins and antioxidant capacity showed some variation during storage, with a differential behaviour of each compound according to the sample.

Keywords: Fresh-cut aromatic herbs; Nutritional evaluation; Antioxidant capacity; Storage; Ready-to-eat salads

7.1 Introduction

Fresh-cut vegetables used in ready-to-eat salads are minimally processed healthy products. This is a growing market that has gained the consumer's preference for its convenience and due to the growing awareness of the benefits of an adequate food intake. An "optimal nutrition" could provide the required amount of nutrients (carbohydrates, proteins, fats, vitamins, and minerals) and also promote health, improve wellbeing and reduce the risk of developing diseases (presence of antioxidant compounds) (Francis et al., 2012; Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2010). Fresh vegetables must be included in a balanced diet and ready-to-eat salad mixtures are an option that can easily fit the busy lifestyle of today's consumers (Tomás-Callejas, Boluda, Robles, Artés, & Artés-Hernández, 2011). The fresh-cut industry is continuously evolving and pursuing new varieties and innovations to meet consumer expectations in terms of convenience, freshness, new flavours and quality (Martínez-Sánchez et al., 2012). One of the latest innovations has been the inclusion of aromatic herbs to add more intense flavour to the salad mixtures. Fresh-cut herbs are minimally processed (washed, cut and packaged at chilling temperatures) vegetables, being the quality of the salad mixtures dependent on the combination of several properties from each product. Besides freshness, that is the principal quality factor of ready-to-eat vegetables, tenderness, safety and uniformity of green colour are also desirable characteristics (Barrett, Beaulieu, & Shewfelt, 2010). Microbiological safety is essential and is one on the most studied quality parameters in these products (Caleb, Mahajan, Al-Said, & Opara, 2013; Jacxsens, Devlieghere, Ragaert, Vanneste, & Debevere, 2003). Nevertheless, nutritional quality is also gaining more attention (Alarcón-Flores, Romero-González, Martínez Vidal, Egea González, & Garrido Frenich, 2014; Fan & Sokorai, 2008).

The use of aromatic herbs in the human nutrition has been described for centuries and has a place in all the cultures. Traditionally, herbs can be used fresh, dried, whole, chopped or ground, and are used to give flavour to food and beverages, reducing the need for salt and fatty condiments (Salgueiro, Martins, & Correia, 2010; Viuda-Martos et al., 2010; Wong & Kitts, 2006). Certain aromatic herbs are also listed as "medicinal plants" and could provide the organism with extra antioxidant compounds, improve digestion, and have some antibacterial, anti-inflammatory, antiviral and anti-carcinogenic activities (Charles, 2013). Phenolic acids, flavonoids, sterols, and coumarins are the most-referred bioactive compounds in aromatic herbs with functional properties (Charles, 2013). Due to their antioxidant and antimicrobial properties, aromatic herbs are also used as

preservative agents, playing an important role in the shelf life conservation of foods and beverages (Salgueiro et al., 2010; Wong & Kitts, 2006).

Minimally processed and individually packed fresh-cut aromatic herbs can be found in market. However, the growth of this sector did not have the success reported for other fresh-cut products, due to the high perishability of these leaves (Luo, McEvoy, Wachtel, Kim, & Huang, 2004). Nevertheless, fresh aromatic herbs retain more aroma than the dried product, which is a clear advantage of these products (Curutchet, Dellacassa, Ringuelet, Chaves, & Viña, 2014). Minimal processing increases the vegetables respiratory rate and can lead to a more promptly onset of senescence signs on the fresh-cut leaves and consequently loss of quality (Junqueira-Gonçalves et al., 2012; Martínez-Sánchez, Allende, Cortes-Galera, & Gil, 2008). Although there are several works concerning the chemical composition and microbiological safety of these herbs (Almeida, Mezzomo, & Ferreira, 2012; Alves-Silva et al., 2013; Hao et al., 2011; Junqueira-Gonçalves et al., 2012; Kamat, Pingulkar, Bhushan, Gholap, & Thomas, 2003; Luo et al., 2004; Ninfali, Mea, Giorgini, Rocchi, & Bacchiocca, 2005; Rosa, Sapata, & Guerra, 2007; Trigo et al., 2009), studies on the stability of the nutritional quality of fresh-cut herbs during shelf life are scarce (Curutchet et al., 2014). Therefore, the main aim of this work was to study the stability of nutritional quality during refrigerated storage of minimally processed chives, coriander, parsley and spearmint leaves, which are within the most used aromatic herbs in the gastronomy of the European countries (Almeida et al., 2012; Hao et al., 2011; Junqueira-Gonçalves et al., 2012; Rosa et al., 2007). The nutritional analysis focused on the evolution of their macronutrient composition, vitamins and minerals content during a storage period. Besides, the antioxidant capacity of the studied herbs was assessed together with the quantification of the phenolic and flavonoids content. This study also monitored several physicochemical characteristics of the leaves as colour, total soluble solids (TSS), total titratable acidity (TTA) and pH evolution during a 10-day storage period.

7.2 Material and Methods

7.2.1 Samples

The minimally processed fresh-cut aromatic herbs were obtained from a producer (Odemira, Portugal) and correspond to chives (*Allium schoenoprasum*), coriander (*Coriandrum sativum*), parsley (*Petroselinum crispum*) and spearmint (*Mentha spicata*) leaves. Due to very different formats and sizes of leaf samples, chives were cut in small pieces, while coriander, parsley and spearmint leaves were packed whole. Upon arrival to

the laboratory, one day after being minimally processed (washed, cut and packed), the fresh-cut aromatic herbs were separated in two different sets. The first set was prepared for quality analyses and the second stored under refrigerated conditions (3 ± 1 ºC) for 10 days. A subsample of fresh leaves from each set (about 200 g), at day 1 and day 10, was used for colour, TSS, TTA, pH and macronutrient determinations. The remaining leaves were freeze-dried (Telstar Cryodos-80, Terrassa, Barcelona), reduced to a fine powder in a knife mill (GM 200, RETSCH, Haan, Germany) and stored protected from light, oxygen and heat until analyses (micronutrients and antioxidant capacity).

7.2.2 Quality analyses

7.2.2.1 Physicochemical characteristics

Leaves color parameters L*, a* and b* were determined with a tristimulus colorimeter (CR-400Chroma Meter, Konica Minolta, Japan), where L* defines the lightness (0<L*<100). Parameters a* define red (+) to green (-) and b*, blue (-) to yellow (+) chromaticity and were used to calculate chroma value ($C^* = (a^{*2} + b^{*2})^{1/2}$). The equipment was set up for illuminant D65 with 10º observer angle and calibrated using a standard white plate. Thirty to forty measurements were made in different leaves at each sampling day. Total soluble solids (TSS) were determined on juice, obtained by grinding the fresh leaves, with a Digital Refractometer (ºBrix, HI 9680, Hanna Instruments, USA). The pH was measured with a pH-meter (Crison Instruments, Barcelona, Spain) in 10 g of leaves homogenized in 20 mL of deionised water (AOAC, 2000). Total titratable acidity (TTA) was determined by titration with 0.1 M NaOH to pH 8.1 (AOAC, 2000) and expressed as mg of citric acid/100g fresh weight (f.w.) product.

7.2.2.2 Nutritional Composition

Water, protein (factor of 6.25), fat, ashes and total dietary fibre contents were determined according to the AOAC (2000) methods, in the samples from the two sets (day 1 and day 10). All values were presented as a percentage, being carbohydrates calculated by difference. All proximate composition analyses were done, at least, in triplicate.

Mineral composition was evaluated by a High Resolution-Continuum Source Atomic Absorption Spectroscopy (HR-CS-AAS) method optimized by Santos, Oliva-Teles, Delerue-Matos, and Oliveira (2014). Briefly, 150 mg of freeze dried sample was digested with 9 ml of mixture of nitric acid/ ultrapure water (2:1) by microwave assisted digestion

(MARS-X, CEM, Mathews, NC, USA). Potassium, sodium, calcium, magnesium, iron, manganese and zinc were analysed with flame atomization (HR-CS-FAAS) (ContrAA 700, Analytik Jena, Germany), while copper was determined in the same equipment but with electrothermal (EAAS) atomization. Phosphorous content was measured according to the 4500-P standard method (Greenberg et al., 1992) in a UV-Vis spectrophotometer. Four replicates of each sample from the two sampling days were analysed. Several free forms of water-soluble vitamins (C, B_1, B_2, B_3, B_5, B_6 and B_9) and fat- soluble vitamins (Pro-vitamin A and E (α-tocopherol)) were assessed by HPLC-MS/MS and HPLC-DAD methods described by Santos, Mendiola, Oliveira, Ibáñez, and Herrero (2012). Briefly, 250 mg of freeze dried sample was extracted with 16 mL of 10 mM ammonium acetate/methanol 1:1 (v/v) in an ultrasound bath for 15 minutes. After centrifugation (14000 g; 15 min) the supernatant was concentrated under nitrogen stream and injected into a HPLC–ESI-MS/MS system (Thermo Scientific, San Jose, CA, USA) to determine the water-soluble vitamin content. The pellet was re-extracted twice with ethyl acetate (0.1% BHT) (6 + 6 mL) in an ultrasound bath (15 min). After centrifuged (14000g, 15 min.) the two supernatants were combined and dried under nitrogen stream. The residue dissolved in 3 mL of ethyl acetate and injected in a HPLC-DAD system (Agilent 1100 Palo Alto, CA, USA) to determine the fat-soluble vitamin content of the samples. Vitamins were also determined in the beginning and at the end of the storage.

7.2.2.3 Determination of soluble phenolic and flavonoid contents

Total soluble phenolic and flavonoid were determined in extracts prepared as described in the previous section for water-soluble vitamin anlyses, with the following modification: the supernatant was filtered through Whatman No. 1 filter paper and stored in amber glass vials at -18ºC until analysis. The extracts were prepared in triplicate for each sample collect at the two sampling days.

The total soluble phenolic content of the aromatic herbs extract was determined according to the Folin-Ciocalteu procedure (Singleton & Rossi, 1965) with minor modifications (Alves et al., 2010). Briefly, 500 µL of the diluted herb extract was mixed with 2.5 mL of the Folin-Ciocalteu reagent (1:10) and 2 mL of a 7.5% $Na_2CO_3.10H_2O$ solution. The mixture was incubated for 15 min at 45º C, followed by 30 min at room temperature, always protected from light exposure. The absorbance was measured at 765 nm (Synergy HT Microplate Reader, BioTek Instruments, Inc., USA). Total soluble phenolics were quantified trough a gallic acid (GA) calibration curve (linearity range: 5 - 100 mg/L, $r > 0.999$) and reported as mg gallic acid /100 g (f.w.) product

Flavonoid content was determined by a spectrophotometric method that monitors the formation of colored flavonoid-aluminum compound (Barroso, Noronha, Delerue-Matos, & Oliveira, 2011). Four mL of ultrapure water and 300 µL of 5 % $NaNO_2$ solution were added to a 1 mL of diluted sample extract. After 5 min, 300 µL of 10 % $AlCl_3$ solution and after 1 min, 2 mL of 1 M NaOH and 2.4 mL of ultrapure water were also added. The absorbance was measured at 510 nm (Synergy HT Microplate Reader, BioTek Instruments, Inc., USA) and catechin was the standard used to quantify the flavonoid content (linearity range: 5−350 mg/L, r > 0.999). Flavonoid content was expressed as mg of catechin /100 g (f.w.) product.

7.2.2.4 Antioxidant capacity assays

The antioxidant activity of the extracts was determined by two different assays in the same extract used for soluble phenolic and flavonoid determinations. The first was a modification of the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical-scavenging assay described by Barroso et al. (2011). The reaction mixture (50 µL of the diluted extract plus 250 µL of a DPPH• ethanolic solution (9.5×10^{-5} M)) was done directly on the well of the 96-well microplate. The reduction of the DPPH• radical was monitored at 517 nm until it reached a stable absorbance value (30 minutes). A calibration curve was prepared with trolox (linearity range: 2.2 to 168.6 mg/L, $r^2 > 0.996$) and results were expressed as milligrams of trolox /100 g fresh product.

The second antioxidant activity assay was based on the ferric reducing antioxidant power assay (FRAP) described by Benzie and Strain (1999) with minor modifications. Briefly, 2.7 mL of FRAP reagent (10:1:1 of 300 mM sodium acetate buffer (pH 3.6): 10 mM TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) solution: 20 mM $FeCl_3.6H_2O$ solution) was added to 90 µL of the aromatic herbs extract and incubated in a water bath at 37 °C for 30 minutes. 300 µL of the reaction mixture were transferred to a 96-well microplate and the absorbance read at 595 nm in Synergy HT Microplate Reader (BioTek Instruments, Inc., USA). FRAP values were obtained by comparing the samples extract absorption with a ferrous sulphate solution calibration curve (linearity range: 150–1500 µM, $r^2 > 0.997$). The results were expressed as µM of ferrous sulphate/100 g fresh product.

7.2.3 Statistical Analysis

Data were expressed as mean ± standard deviation and the differences among samples and sampling days were tested using one-way ANOVA followed by post-hoc Tukey HSD test. Normal distribution of data in the different samples was assessed by

Kolmogorov–Smnirnov test. Statistical significance was defined as $p<0.05$ (95% confidence level). The statistical analyses were carried out using the Statistica 8.0 software (Statsoft Inc., Tulsa, USA).

7.3 Results and Discussion

7.3.1 Evolution of physicochemical characteristics

The physicochemical characteristics studied (colour, TTS, pH and TTA) monitored the changes in some sensory quality features of the product (colour/appearance and flavour) that have significant influence in the evaluation of the product made by consumers (Barrett et al., 2010). The colour evolution of the fresh-cut aromatic herbs during storage (see Figure 7.1) showed a variation lower than 10% in all parameters evaluated.

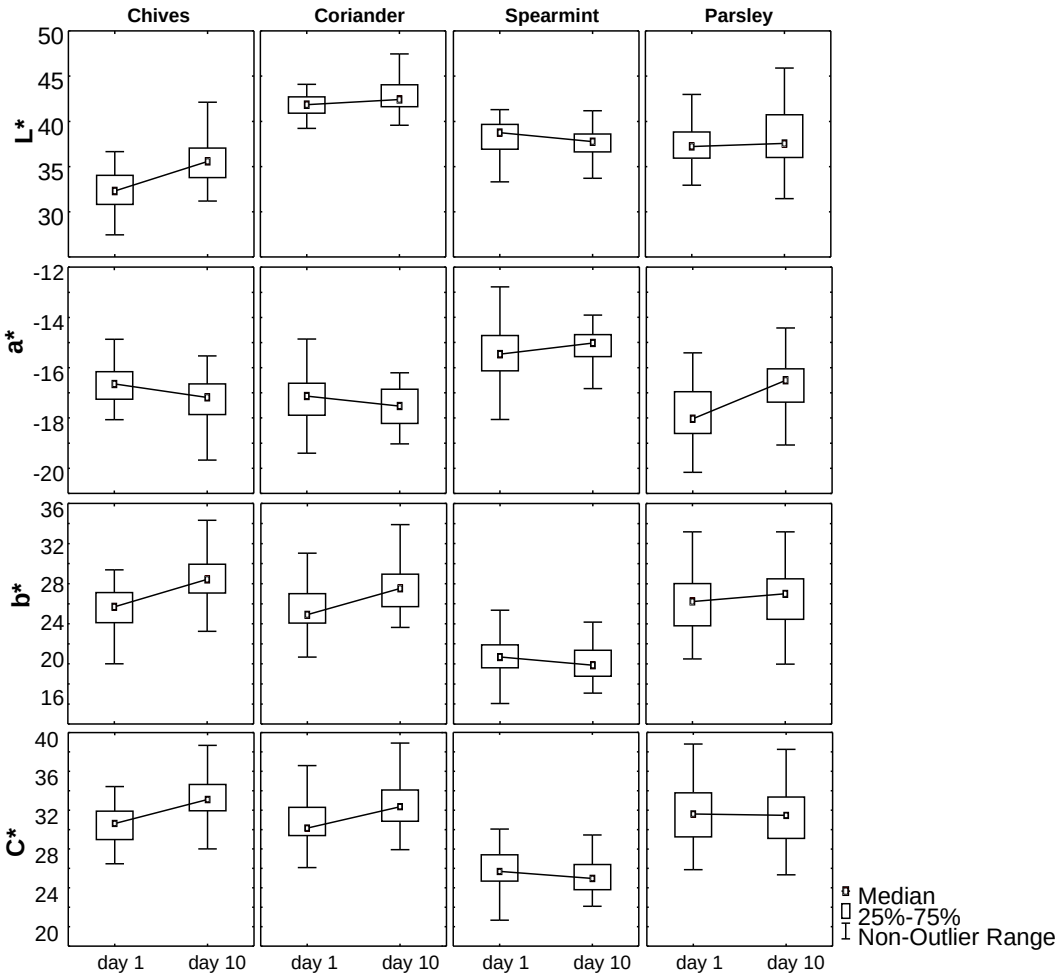

Figure 7.1 Evolution of colour parameters (L*, a*, b*and C*) of the fresh-cut aromatic herbs during storage period.

In general, all leaves maintained a fresh green appearance. No significant (p<0.05) changes were recorded on spearmint leaves, showing a better preservation of their aspect during refrigerated storage. On the other hand, fresh-cut chives, underwent some significant (p<0.05) colour changes, showing a more intense variation (10%) in the b*, L* and C* values, reflecting the appearance of a yellowish tonality (higher b* values) and also a more intense colour of all the leaves (higher L* and C* values). These colour changes in chives have been previously observed (Brueckner & Perner, 2006; Junqueira-Gonçalves et al., 2012; Viña & Cerimele, 2009). This behaviour could limit the product shelf life, diminishing the product quality to the consumer's eyes. Coriander leaves also showed a significant (p<0.05) increase of the b* values, while in parsley a slight loss of the green intensity (higher a* values) was registered.

The changes occurred on the TTS, pH and TTA values are summarized in Table 7.1. The data suggest a slight evolution of the flavour of these fresh-cut aromatic herbs during storage (Barrett et al., 2010). In most cases, differences between the first and the last day of storage were statistically significant (p<0.05), although, it is worth mention that were lower than 10% of the initial value. Exceptions were registered in the evolution of the TTS of parsley and spearmint leaves that rose 23.9% and decreased 31.3%, respectively. TTA of chives and coriander increased 27.7% and 13.8%, respectively).

Table 7.1 Variation of physicochemical parameters through the storage period (mean value ± standard deviation; * means no significant variation between sampling days (p<0.05); TSS: total soluble solids; TTA: Total titratable acidity).

	TSS (%)	pH	TTA(%)[1]
Chives			
day 1	4.60 ± 0.08	6.01 ± 0.01	0.12 ± 0.01
day 10	4.78 ± 0.05	5.96 ± 0.00	0.16 ± 0.01
Coriander			
day 1	6.38 ± 0.22*	6.20 ± 0.04	0.21 ± 0.01
day 10	6.48 ± 0.05*	6.11 ± 0.01	0.24 ± 0.01
Spearmint			
day 1	6.88 ± 0.13	6.54 ± 0.01	0.16 ± 0.01
day 10	4.73 ± 0.10	6.57 ± 0.01	0.15 ± 0.01
Parsley			
day 1	7.53 ± 0.05	5.96 ± 0.01	0.26 ± 0.01*
day 10	9.33 ± 0.26	6.20 ± 0.01	0.28 ± 0.02*

[1] % of citric acid

7.3.2 Nutritional Composition

The overall macronutrient composition of the leaves is presented in Figure 7.2. In general, macronutrients were stable during storage (10 days under refrigerated conditions). The only differences between sampling days were very small and were registered in water and dietary fibre content of parsley, where losses of 1.4% and 2.0%, respectively, where found.

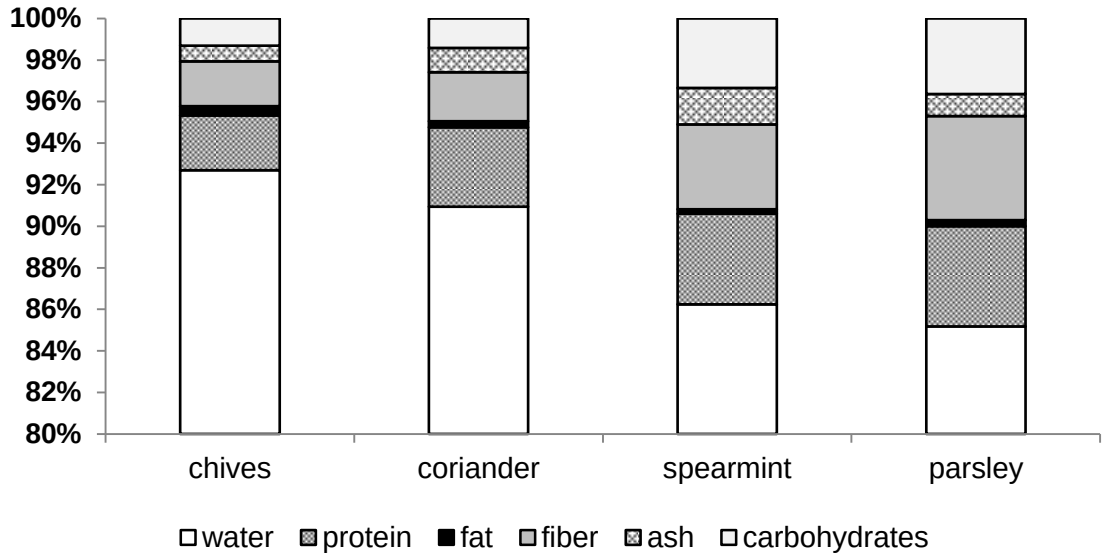

Figure 7.2 Macronutrients composition of the fresh-cut aromatic herbs (carbohydrates calculated by difference).

All samples showed a characteristic ($p<0.05$) nutritional composition. Chives and coriander had higher water content than parsley or spearmint (91-92% versus 85-86%), while these later showed a higher protein content (4-5%). As expected, a very low fat content (0.21% in spearmint, 0.29% in coriander and parsley and 0.45% in chives) was found in the aromatic herbs. On the other hand, a high dietary fibre content was found in the samples (between 2 and 5%) when compared with other leaves normally used in ready-to-eat salads, like lettuce, watercress or spinach (Martins, 2010). This seems to be one of the nutritional advantages of including aromatic herbs in ready-to-eat salads. Some of the health benefits attributed to the consumption of high fibre diets are the ability to decrease cholesterol and the risk of coronary heart diseases, reduced type II diabetes and improve weight maintenance (EFSA, 2010).

The mineral content was stable along the storage period, as nearly all variations were less than 10% of the initial value. This behaviour was expected because minerals

are normally not metabolized during the storage period (Sánchez-Mata, Cámara, & Díez-Marqués, 2003). Table 7.2 shows the mean values of the mineral content determined in the samples from the two sampling days (day 1 and day 10). Coriander had the highest mineral content (813.7 ± 26.7 mg/100g f. w.), followed by parsley and spearmint leaves (794.5 ± 47.1 mg/100g f.w. and 783.3 ± 25.1 mg/100g f.w., respectively), while chives (494.6 ± 29.3 mg/100g f.w.) had the lowest mineral content. The mineral composition indicated that aromatic herbs may be a good source of potassium, phosphorous and calcium. Parsley had a sodium content 6 times higher than the others samples, while spearmint had more iron and manganese than the other aromatic herbs. The mineral content could be affected by several factors, like genetics, soil and weather conditions and agronomic practices (Khader & Rama, 2003; Sanchez-Castillo et al., 1998), being expected that the values presented could differ in some extent from others reported in the literature for the same herbs (Martins, 2010; Singh, Kawatra, & Sehgal, 2001). However, the mineral profile found in these aromatic herbs followed the profile reported for other green leafy vegetables (higher concentration of K, P and Ca) (Santos et al., 2014).

Table 7.2 Mineral content composition (mean values in mg/100 g fresh weight ± standard deviation; n=8)

	Chives	Coriander	Spearmint	Parsley
P	77.39 ± 8.05	146.77 ± 5.48	128.03 ± 5.52	162.97 ± 28.69
K	317.44 ± 15.33	509.76 ± 17.03	439.37 ± 10.54	222.09 ± 10.83
Na	4.84 ± 0.35	19.04 ± 0.54	20.37 ± 0.83	125.46 ± 3.01
Ca	69.82 ± 4.20	87.09 ± 1.94	125.79 ± 5.46	183.98 ± 2.63
Mg	23.62 ± 1.32	48.58 ± 1.65	63.51 ± 2.42	95.68 ± 1.75
Fe	0.58 ± 0.03	1.33 ± 0.04	3.68 ± 0.22	2.82 ± 0.11
Mn	0.44 ± 0.01	0.59 ± 0.02	1.90 ± 0.04	0.63 ± 0.02
Zn	0.36 ± 0.01	0.44 ± 0.03	0.46 ± 0.03	0.86 ± 0.07
Cu	0.06 ± 0.00	0.10 ± 0.00	0.15 ± 0.01	0.08 ± 0.00

(Mineral elements abbreviations: P: phosphorous; K: potassium; Na: sodium; Ca: calcium; Mg: magnesium; Fe: iron; Mn: manganese; Zn: zinc; Cu: copper)

Regarding vitamin contents, aromatic herbs showed higher levels of vitamin C, vitamin E and pro-vitamin A than the other water-soluble vitamins analysed (thiamine, B_1; riboflavin, B_2; nicotinamide, B_3; pantothenic acid, B_5; pyridoxine, B_6; and folic acid, B_9) (see Table 7.3). Chives had the highest vitamin C and vitamin E contents at day 1, while spearmint revealed the lowest concentrations of these micronutrients. Within the B

vitamins, the lowest values correspond to the pyridoxine (B_6) and thiamine (B_1) concentrations.

Table 7.3 Fat- and Water-soluble vitamins found in fresh-cut aromatic herbs (mean value ± standard deviation relative to fresh weight (f.w.); * indicates no significant variation ($p<0.05$) between sampling days).

Vitamins	Chives	Coriander	Spearmint	Parsley
α-tocopherol (E)		**mg/100g f.w.± sd**		
day 1	2.8 ± 0.1	2.6 ± 0.1	1.4 ± 0.0*	2.3 ± 0.1
day 10	1.8 ± 0.1	2.1 ± 0.1	1.5 ± 0.2*	2.1 ± 0.0
β-carotene (pro vit.- A)		**mg/100g f.w.± sd**		
day 1	12.1 ± 0.4	16.2 ± 0.1	10.6 ± 1.1	11.6 ± 0.3
day 10	9.4 ± 0.3	15.2 ± 0.2	8.8 ± 0.0	16.8 ± 0.6
Ascorbic Acid (C)		**mg/100g f.w.± sd**		
day 1	93.1 ± 2.3	47.9 ± 6.0	0.5 ± 0.0	59.2 ± 4.0
day 10	1.3 ± 0.1	1.8 ± 0.3	0.3 ± 0.0	0.8 ± 0.2
Thiamine (B_1)		**µg/100g f.w.± sd**		
day 1	69.0 ± 4.7	52.7 ± 2.3	121.4 ± 10.4	47.3 ± 3.7
day 10	104.1 ± 5.7	61.4 ± 3.9	216.2 ± 13.0	97.1 ± 8.2
Riboflavin (B_2)		**µg/100g f.w.± sd**		
day 1	170.2 ± 16.7*	176.8± 23.1	169.1± 25.7*	196.9± 9.3
day 10	182.2 ± 5.7*	102.6± 3.6	143.7± 13.7*	251.2± 5.3
Nicotinamide (B_3)		**µg/100g f.w.± sd**		
day 1	235.8 ± 25.2	214.3 ± 10.9*	281.6 ± 7.2	298.8 ± 28.9*
day 10	387.6 ± 14.2	218.0 ± 5.2*	238.9 ± 13.4	283.8 ± 12.8*
Pantothenic Acid(B_5)		**µg/100g f.w.± sd**		
day 1	112.4 ± 11.6	1296.9 ± 28.3	557.0 ± 31.1	630.1 ± 73.9
day 10	269.4 ± 6.8	1930.3 ± 194.7	1621.7 ± 117.9	1335.6 ± 296.4
Pyridoxine (B_6)		**µg/100g f.w.± sd**		
day 1	5.0 ± 1.3	5.3 ± 0.1	8.4 ± 0.9	24.2 ± 1.5
day 10	11.0 ± 0.6	14.6 ± 0.6	4.8 ± 0.1	37.5 ± 6.3
Folic Acid (B_9)		**µg/100g f.w.± sd**		
day 1	n.d.	n.d.	n.d.	6.03 ± 1.45*
day 10	n.d.	n.d.	n.d.	7.37 ± 1.61*

The free form of vitamin B_9 (folic acid) was only detected in parsley (mean content of 6.70 ± 1.6 µg/100g f.w.), being this value lower than the reported in the literature (Martins, 2010). This difference can be justified by the methodology followed that did not include the

enzymatic extraction, used for the total quantification of folates in food (Phillips, Ruggio, Ashraf-Khorassani, & Haytowitz, 2006; Santos et al., 2012). The vitamins were the micronutrients with the highest variation during storage, being the changes more intense in the water soluble vitamins than in vitamin E and pro-vitamin A (Table 7.3). Vitamin C was the principal vitamin, representing 85.8%, 70.0% and 79.7% of the total vitamin content of chives, coriander and parsley samples from day 1, respectively. However, this was also the vitamin with more losses (96-98%) in the storage period, probably caused by the oxidation to dehydroascorbic acid, as a defence reaction against oxygen species, frequently described in fresh-cut vegetables (Bergquist, Gertsson, & Olsson, 2006; Reyes, Villarreal, & Cisneros-Zevallos, 2007; Tomás-Callejas et al., 2011). In the work presented by Tomás-Callejas et al. (2011), 95% of the vitamin C present in a minimally processed vegetable was in the reduced form.

Concerning the B vitamins evolution, the behaviour of each vitamin varied strongly among samples, being detected higher levels of vitamins B_1, B_5 and B_6 in some samples stored under refrigeration. Pantothenic acid (B_5) levels increased greatly during storage (187.5% in spearmint; 139.7% in chives; 111.9% in parsley; and 48.0% in coriander), being this behaviour common to other minimally processed vegetables (Santos et al., 2012). The changes in the vitamin contents of these herbs seem to be related to the plant normal defence metabolism against oxidative stress (e.g., vitamin C). Other hypothesis is related to the action of some enzymes against a conjugated form of the vitamin (e.g., release of pantothenic acid from coenzyme A (CoA)) or resulting from post-harvest synthesis (Hounsome, Hounsome, Tomos, & Edwards-Jones, 2009; Sánchez-Mata et al., 2003). Even a microbial origin should not be discard, due to microbial growth that occurs in minimally processed vegetables during storage (M. I. Santos et al., 2012).

7.3.3 Total soluble Phenolic and Flavonoid contents

The total soluble phenolic and flavonoid contents of the aromatic herbs are presented in Figure 7.3. All samples showed a different content of soluble phenolics, with spearmint and parsley showing the highest values and chives the lowest. These results agree with those reported by Wong and Kitts (2006) about a higher phenolic content in parsley than in coriander leaves. Regarding flavonoid content, it was also higher in spearmint, but, unlike to total soluble phenolics, the flavonoid levels found in parsley extracts were, together with chives, the lowest values found the studied aromatic herbs. These two parameters also had a different evolution during storage. The flavonoids were stable in chives and parsley, and suffered a 28.8% and 12.1% decrease in coriander and

spearmint, respectively, between the two sampling days. These results agree with works that described flavonoids as more stable during storage than other phenolic acids (DuPont, Mondin, Williamson, & Price, 2000; Martínez-Sánchez, Marín, Llorach, Ferreres, & Gil, 2006). The total soluble phenolic content increased during storage on chives (20.4%), parsley (16.2%) and spearmint (9.8%) leaves, showing a decrease in coriander. The evolution of the phenolic content in fresh-cut vegetables during storage varies with plant species and minimal treatment procedure (Alarcón-Flores et al., 2014). The wounding inductive stress caused by cutting leaves can lead to the production of phenolic compounds in response to the stress caused by the release of certain enzymes from the cell cytoplasm. However, the presence of other compounds, like vitamin C, can justify the stability of phenolic content during storage (Reyes et al., 2007).

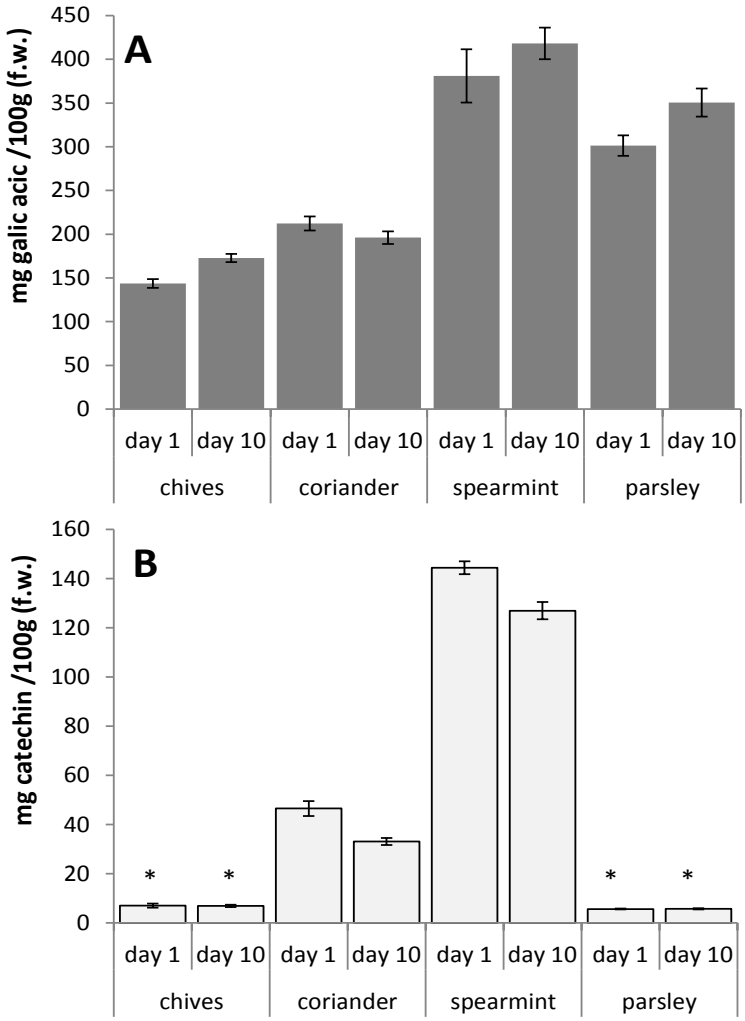

Figure 7.3 Total soluble phenolic (A) and flavonoid (B) contents evolution during storage (* indicates no significant variation ($p<0.05$) between sampling days).

7.3.4 Antioxidant capacity

Both antioxidant capacity assays applied to the aromatic herbs extracts revealed a high antioxidant activity in spearmint and lower in the other three herbs evaluated (Figure 7.4 A and B).

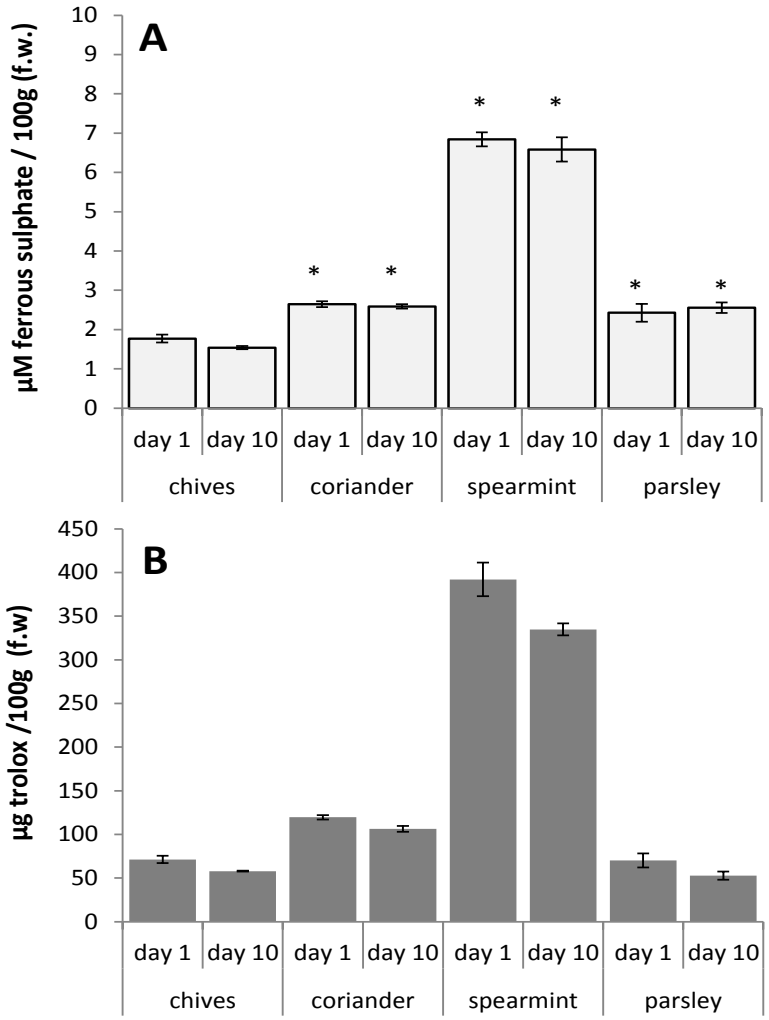

Figure 7.4 FRAP (A) and DPPH (B) antioxidant capacity assays results (* indicates no significant variation ($p<0.05$) between sampling days).

The antioxidant capacity of vegetables is normally associated to a high content of phenolic compounds, and also vitamin C, vitamin E and carotenoids. In this work, the highest antioxidant activity occurred in the sample with the higher phenolic content, showing that these compounds could be accountable for the antioxidant capacity in these leaves. The lower flavonoid contents of chives and parsley corresponded also to the lowest antioxidant capacity determined using both assays. The antioxidant capacity of the extracts at day 1 and at day 10 behaved differently in the different assays. The DPPH• scavenging activity diminished a ($p<0.05$) 24% in parsley, 18% in chives, 14% in

spearmint and 11% in coriander. In the other hand, the capability to reduce the Fe^{3+}-TPTZ complex only showed some decrease in chives (-13%) (Figures 7.4 A and B). The differences found between these methods are related to the different chemical mechanisms of the methods.

The high content of phenolic compounds in aromatic herbs and the small differences in the antioxidant capacity along the storage indicate that few grams of these herbs in the salad can represent a way to increase the antioxidant capacity of the daily diet with possible health benefits (Ninfali et al., 2005). Additionally, antimicrobial activities were attributed to some phenolic compounds, which may act as stabilizer agents when the aromatic herbs are added to other food products, playing an important role in their shelf life (Salgueiro et al., 2010).

7.4 Conclusion

From a global perspective, the inclusion of fresh-cut aromatic herbs as ingredients of ready-to-eat salads appears to be a good choice, taking into account the increase of nutritional value and diversity of flavours. This strategy can avoid the use of salad dressings, normally containing high sodium, sugar and fat contents to improve the salad flavour. The quality of the studied aromatic herbs was, in general, stable during the storage period (10 days), showing only small changes in their phytonutrient content. The fresh-cut aromatic herbs revealed to be a good source of dietary fibre, minerals (especially potassium and calcium), fat soluble vitamins E and A and also of some water soluble vitamins like nicotinamide (B_3) and pantothenic acid (B_5). The presence of antioxidant compounds in the diet, like the phenolic acids and flavonoids, could also be enhanced with the incorporation of these herbs.

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CHAPTER 8.

Final Remarks

This chapter presents the major conclusions and achievements of this thesis

8.1 Final Remarks

The research planned and executed during the development of this PhD thesis, had always in mind the need to obtain information that could support the consumers' choice for fresh-cut baby leaf vegetables. The main objective of the work focused, mainly, on the evaluation of the nutritional quality of 12 minimally processed baby leaf vegetables and 4 fresh-cut aromatic herbs during storage (10 days), to determine the main nutrients and phytonutrients that consumers can get from these vegetables during shelf life. Another aspect that this thesis tried to answer was related to the nutritional quality of these products when close to the end of shelf life. This information is also important for producers, as nutritional composition is becoming more common in the labels of minimally processed vegetables.

As leafy vegetables normally consumed in salads in a raw state, the nutritional quality of these products is principally related to their vitamin, mineral, phenolic and carotenoid contents. In this thesis, new analytical methods were presented and discussed with a focus on the nutritional changes that can affect the postharvest quality of leafy green vegetables. The use of simple, effective and "greener" extraction methods was privileged during the development of analytical methodologies. The extraction methods proposed in this work also aim to be applied to all 16 species of green leafy samples studied, showing that they can be appropriate to be used in other similar vegetable matrices. Specifically regarding the extraction of nutritional and bioactive compounds, the main contributions of this thesis were:

- The sequential ultrasound based extraction methodology applied to obtain several free water-soluble vitamins (vitamins C, B1, B2, B3, B5, B6 and B9), fat-soluble vitamins (vitamin E and provitamin A) and carotenoid compounds. This method allowed extracting from the same sample a whole range of compounds with very different chemical structures, with recoveries tests ranging from 83 to 105%.
- An optimized microwave digestion procedure that was successfully applied to mineralize different baby leaf vegetables. With the proposed digestion program, the vegetable samples were completely digested within one hour and the use of a diluted nitric acid showed to be more effective in the conditions selected. This method provided recoveries ranging from 91 to 110%.
- The Pressurized Liquid Extraction (PLE) method optimized, for the first time, to extract phenolic compounds from baby leaf vegetables. The procedure revealed

to be an efficient and “green” option to extract a wide range of polyphenols from different green leafy vegetables. The method potentiates the solubility of the phenolics in the liquid solvent (MeOH 70%) due to the use of a high pressure and temperature.

Due to the normal low range of bioactive compounds in these matrices (within a few milligrams or micrograms/ 100g of fresh vegetable), powerful analytical techniques were chosen. For compounds like vitamins, phenolics and carotenoids, coupling HPLC methods with diode array and mass detectors represent an analytical advantage. The high resolution detector used in mineral analysis also revealed to be advantageous for an accurate routine mineral analysis. Regarding the detection, identification and quantification of the different bioactive compounds evaluated in this thesis, the main contributions were:

- The proposed HPLC-ESI-MS/MS method to identify and quantify free forms of water-soluble vitamins (vitamins C, B1, B2, B3, B5, B6 and B9). The elution gradient used for separate the different vitamins was optimized, and the final method attained low LODs (0.1-42.3 ng/ml) and LOQs (0.2-128 ng/ml) for each vitamin and also an appropriate intra-day and inter-day precisions.
- The HPLC-DAD method applied in the determination of the major fat-soluble vitamins (vitamin E and provitamin A) present in green leafy vegetables. This method was developed simultaneously with the method presented for the analysis of water-soluble vitamins, and also showed suitable LOD and LOQ and appropriate intra-day and inter-day precisions for the intended purpose. For fat-soluble compounds a different analytical column (C30) showed to be more appropriate, and the DAD detector was sensitive enough for the range of these compounds in leafy vegetables.
- The HR-CS-AAS method used to quantify macro and micro minerals in leafy vegetable matrices. This method showed to be simple, accurate and with less preparation steps than the conventional AAS methods. The method was also further validated by the analysis of a certified reference material.
- The HPLC-DAD-ESI-MS^n method used for identification and quantification of more than 200 phenolic compounds in 11 baby leaf vegetables. The phenolic profile of each sample was successfully resolved with the HPLC conditions selected, showing to be suitable for different green leafy samples. The tentative identification of a large number of different phenolic compounds only was

possible by combining the UV-Vis spectra information with MS spectra data and fragmentation patterns analysis from native and hydrolysed extracts.

- The HPLC-DAD-APCI-MS^n system proposed for the identification of the carotenoids present in green leafy vegetables. Coupling the HPLC-DAD method (previously applied to determination of fat-soluble vitamins) to a mass detector allowed the identification of the major carotenoid compounds, in cases where the UV-Vis spectra of the compound could not permit an undoubtedly identification. Combining the information of both spectra (UV-Vis and MS and MS^2) was also essential to distinguish between carotenoids that had the same protonated molecule.

Regarding the nutritional quality of all samples analysed, they all show different contents of the analysed compounds. The influence of the genetic factor on their nutritional composition, especially on their vitamin, mineral, phenolic and carotenoid content, was evident. Each species and also variety, revealed their own metabolic profile. Their phytonutrient composition, especially the phenolic content, was variable within the different samples. This clearly points to major advantages in consuming several green leaf varieties instead of only one or two species. Choosing different leaves, adds diversity of phytonutrients that could represent different health benefits and, at the same time, improves the visual appearance (more colour and leaf formats) and flavour of a salad mixture. However, as green leafy samples, there were some common aspects within the majority of the analysed samples. They showed a higher content of vitamin C, provitamin A and vitamin E than the B group vitamins. The mineral content also followed the same profile, showing high levels of potassium and calcium, and iron as the principal micro mineral of their composition.

The effect of the maturity stage is one of the key aspects when studying the quality of baby leaf products, due to their higher metabolism rates. The nutritional quality of the baby leaves studied did not show any disadvantage comparing to more mature products, showing always similar results to the ones reported to more mature minimally processed samples. The baby leaf did not represent a smaller intake of nutrients in relation to more mature samples; with the advantage that they could add more diversity of leafy vegetables to the diet. As showed in chapter 6 and 7 of this thesis, introducing new baby leaf products, as pea shoots, and adding aromatic herbs to a baby leaf salad mixture could enhance the content of bioactive compounds (particularly antioxidant compounds) of a meal.

Relatively to the stability of the nutritional quality of the studied baby leaf samples, water-soluble vitamins and some phenolic compounds showed the greatest variations during storage, within all evaluated nutritional quality parameters. The behaviour of the different compounds was also very dependent on the species analysed, being some species more susceptible to lose vitamins and others to show greater changes in the phenolic content (in particular hydroxybenzoic and hydroxycinnamic acids). However, it should be mentioned that in most cases, those changes were small and the stability found in the others parameters evaluated point to the maintenance of the overall nutritional quality of the baby leaf during the 10 days storage period considered.